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Capsaicin-sensitive mechanisms in the modulation of rat colonic vascular permeability under physiological and pathological conditions

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Summary — Inflammatory bowel disease (IBD) causes a prolonged life-quality reduction of patients and high costs for health services. The aim of this study was to explore the possible involvement of peptidergic capsaicin-sensitive afferent nerves (CSN) in the pathogenesis of IBD. For the defunctionalization of colonic CSN, the lower part of the colon (1–4 cm from the anus) was exposed through a midline laparotomy and small pieces of gelfoam moistened with a solution of capsaicin (1%, 100 µL) was applied onto the serosal surface for 30 min in male Wistar rats. Colonic vascular permeability was assessed by measuring the extravasation of [^{125}I] human serum albumin (2 µCi/kg, iv, 2 h prior to killing). Two months after capsaicin treatment a significant increase in albumin extravasation was found in the lower ($P < 0.005$), but not in the upper (5–8 cm from the anus) part of the colon as compared to the sham-operated control. Intrarectal (8 cm from anus) administration of trinitrobenzene-sulphonic acid (TNBS; 30 mg/rat) induced similar plasma leakage in the lower and upper colon of control (CSN-intact) rats ($P < 0.001$) 1 week later. TNBS + ethanol (50%) produced further extravasation throughout the colon ($P < 0.001$) of CSN-intact animals. In the lower colon of capsaicin-pretreated rats TNBS-alone provoked an increase in plasma extravasation ($P < 0.001$) similar to that caused by TNBS + ethanol in CSN-intact rats. In the upper colon there was no difference in the effect of TNBS-alone on plasma leakage between control (CSN-intact) and CSN-depleted rats. The results suggest that capsaicin-sensitive nerves may play a significant protective/anti-inflammatory role in the colon under normal and pathological conditions.

capsaicin-sensitive innervation / inflammatory bowel disease / vascular permeability / vascular endothelium / sensory neuropeptides

Introduction

Inflammatory bowel disease (IBD) is the collective term used to describe two gastrointestinal disorders: ulcerative colitis and Chron's disease. Although these diseases have distinctive pathophysiological characteristics, they are frequently considered together because of several clinical and therapeutic similarities (Riis, 1990). Implication of a causal factor remains elusive, and there is no known cure (Riis, 1990; Hay and Hay, 1992).

Capsaicin-sensitive sensory nerves play an important role in the maintaining of the structural and functional integrity of the gastrointestinal mucosa (Holzer, 1988). There is ample evidence that this effect is mediated by neuropeptides released from afferent nerve endings. Capsaicin selectively stimulates these nerves and causes the release of vasoactive peptides. Hence, mucosal application of capsaicin at low concentrations may effect mucosal protection by releasing peptides which increase mucosal blood flow (Holzer and Sametz, 1986; Holzer, 1988, 1991). In contrast, systemic administration or local application of capsaicin at higher concentrations results in a selective destruction of capsaicin-sensitive afferent neurons (for a review see Jancsó, 1992). Thus, pretreatment with capsaicin may lead to aggravation of tissue damage. Available experimental evidence indicates that sensory nerves may affect the development of colonic IBD (Evangelista and Meli, 1989; Reinshagen *et al*, 1994).

The present experiments were initiated in an attempt to reveal possible participation of capsaicin-sensitive sensory nerves in changes of colonic vascular permeability under physiological and pathological conditions.

Part of this work has been presented to the 6th Annual Meeting of the European Neuropeptide Club in Pécs, Hungary, 16–19 June 1996 (Szepes *et al*, 1996).

Materials and methods

Albumin leakage

Male Wistar rats weighing 220–250 g were used. The animals were fasted for 24 h, but received water *ad libitum* before any experiment. For the determination of the vascular albumin leakage as an index of endothelial injury, rats were anaesthetized with ether and ^{125}I-labeled human serum albumin ([^{125}I]I-HSA) was administered (2 µCi/kg) *via* a tail vein. In all experiments, the radiolabeled albumin was administered 2 h before killing. Under ether anesthesia, blood

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(1 mL) was collected from the abdominal aorta in tubes containing trisodium citrate (final concentration 0.318%) and centrifuged (10 000 *g*, 10 min, 4°C). Following cervical dislocation, segments (1.5–2 cm) of the upper (5–8 cm from the anus) and lower part (1–4 cm from the anus) of the large intestine were removed. The [^{125}]I-HSA content of tissue samples and aliquots of blood plasma (100 µL) was determined with a gamma-spectrometer (Nuclear Enterprises NE 1600). The organs were then opened, cleaned, dried out at 100°C for 48 h and weighed. The total radiolabeled albumin content in the intestinal tissue was calculated. Control values were subtracted from the treated values and the data were expressed as the content of plasma in albumin equivalents (µL/g dry tissue).

Defunctionalization of colonic capsaicin-sensitive neurons (CSN-depletion)

Rats were anaesthetized with urethane (1 g/kg, ip). A small piece of gelfoam was moistened with capsaicin (1%, 100 µL; Sigma) and placed onto the serosal surface of the lower part of the colon (1–4 cm from the anus) for 30 min. Sham-operated rats were treated with the solvent of capsaicin. For the determination of vascular permeability changes, tissue samples of the lower and upper colon were removed 2 months later. This examination period was chosen, since among capsaicin-treated (CSN-depleted) rats a moderate diarrhea occurred at this postoperative survival time. The sham-operated group did not show this symptom of inflammation.

Induction of colitis in CSN-depleted and CSN-intact rats

Colonic inflammation was induced with trinitrobenzenesulfonic acid (TNBS) according to the method of Morris *et al* (1989). TNBS (30 mg/rat) or/and ethanol (50%) were administered through the anus *via* a plastic tube advanced 8 cm into the colon of CSN-depleted or control (CSN-intact) rats. Plasma leakage in the upper and lower colon were determined in TNBS-alone and TNBS + ethanol groups 1 week later.

Statistical analysis

The data are expressed as mean ± SEM. For statistical comparisons the Mann-Whitney non-parallel U-test was used; $P < 0.05$ was taken as statistically significant difference.

Results

Effect of CSN-depletion on colonic albumin leakage

CSN-depletion in the lower colon led to a significant plasma leakage ($P < 0.005$, $n = 8$) after 2 months. There was no difference in albumin extravasation in the lower colon of sham-operated animals as compared to the intact control group ($n = 5$ and 8, respectively). In CSN-depleted and sham-operated animals, albumin extravasation in the upper colon was unaffected (fig 1).

Effect of CSN-depletion on albumin leakage during colitis

In CSN-intact control rats administration of TNBS alone induced significant albumin extravasation in the upper and lower colon after 1 week ($P < 0.001$, $n = 5$). In contrast, in CSN-depleted rats TNBS alone produced a more marked increase in vascular permeability in the lower colon ($P < 0.001$, $n = 5$), while in the upper colon there was no difference in

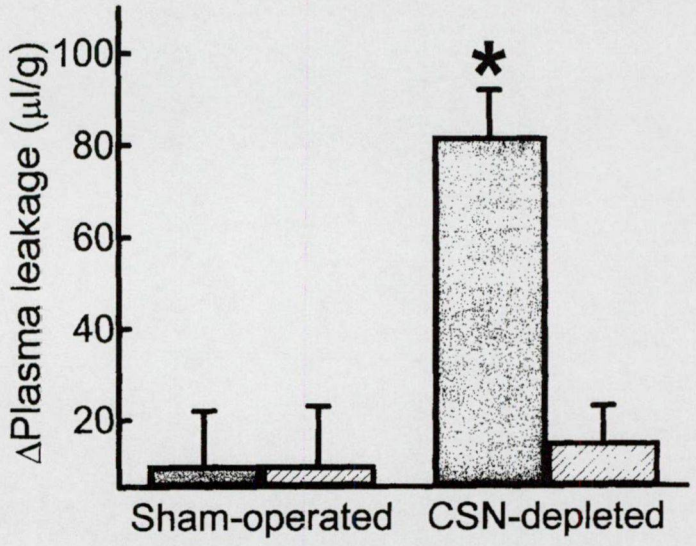

Fig 1. Plasma leakage in the lower part (1–4 cm from the anus; gray column) of the rat colon induced by local capsaicin-sensitive nerve-depletion (CSN-depleted). The plasma leakage values in the upper part of the colon are demonstrated on hatched columns. Plasma leakage was expressed as Δ µL plasma/g dry tissue. Data are shown as means ± SEM of at least five experiments for each group, where statistical significant increase is shown as $*P < 0.05$.

albumin extravasation between CSN-depleted and CSN-intact rats following the administration of TNBS-alone (n = 5 and 8, respectively).

In CSN-intact rats administration of TNBS and ethanol caused marked plasma leakage in the upper and lower colon ($P < 0.001$, n = 5). In CSN-intact animals there was no significant difference in the enhancement of vascular permeability between the upper and lower parts of the colon. In the lower colon of CSN-intact rats TNBS + ethanol brought about similar increase in plasma leakage as TNBS alone in CSN-depleted animals (fig 2).

Discussion

The present findings showed that local CSN-depletion led to a sustained increase in vascular permeability of the colon in control rats. Moreover, in CSN-depleted rats administration of TNBS produced a further increase in albumin leakage which was similar in magnitude to that found after the application of TNBS + ethanol in the lower colon of CSN-intact animals. On the basis of these observations it appears that CSN are involved in the modulation of colonic vascular permeability under physiological and pathological circumstances.

Our results are in agreement with previous findings showing that defunctionalization of extrinsic CSN by systemic administration of capsaicin augmented immune-complex-induced colitis in rabbits (Reinshagen *et al*, 1994), rat colonic inflammation provoked by acetic acid (Eliakim *et al*, 1995) and TNBS + ethanol-induced mucosal injury in the rat colon (Evangelista and Meli, 1989). It has been also demonstrated that local activation of CSN by topical application of capsaicin protects against an acute colonic injury provoked by TNBS + ethanol in rats, an effect which is reversed by a competitive antagonist of capsaicin (Goso *et al*, 1993). Taken together, these results suggest a protective role for CSN in experimental models of IBD.

It is known that CSN contain numerous neuropeptides (Holzer, 1991). The release of these neuropeptides may be responsible for the protective function of this neuronal system in IBD. During induction of colitis by acute immune complex exposure in rabbits, an early reduction of the two most important CSN neuropeptides, *ie* substance P (SP) and calcitonin gene-related peptide (CGRP) has been found (Eysselein *et al*, 1991). These findings indicate that these peptides are released during inflammation. Because of its pharmacological actions (Holzer, 1991; Whittle, 1993) and of its predominant existence in the CSN of gastrointestinal tissues (Mulderry *et al*, 1988; Reinshagen *et al*, 1994), CGRP seems to be the most important peptide involved in the protective function of CSN in experimental models of IBD. Intravenous or intra-arteriolar infusion of CGRP increases resting gastric mucosal blood flow (Dipette *et al*, 1987; Holzer and Guth, 1991; Whittle *et al*, 1992). In contrast, local infusion of the CSN neuropeptides, SP and neurokinin A did not elevate resting mucosal blood flow (Holzer and Guth, 1991). Therefore,

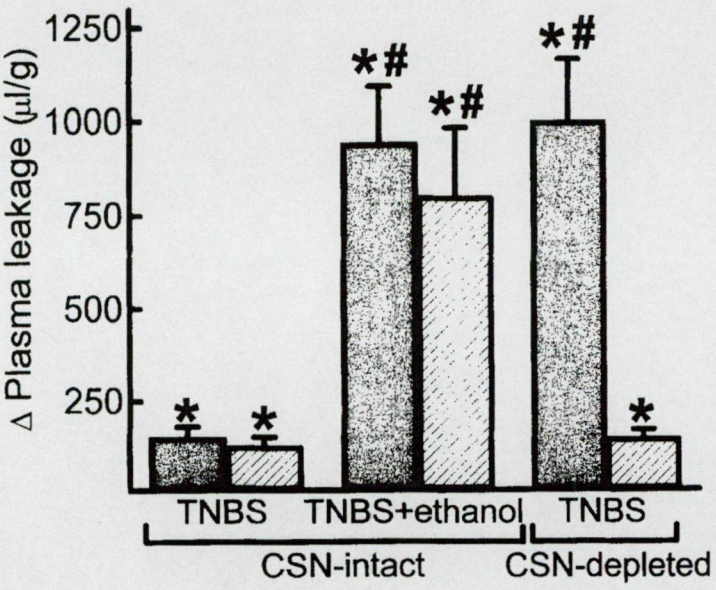

Fig 2. Colonic plasma leakage 1 week after intrarectal (8 cm from the anus) administration of trinitrobenzenesulphonic acid (TNBS, 30 mg/rat) or/and ethanol (50%) in lower (1–4 cm from the anus; gray column) and upper (5–8 cm from the anus) of the colon following capsaicin-sensitive nerve-depletion (CSN-depleted) or in control (CSN-intact) rats. Plasma leakage was expressed as Δ μL plasma/g dry tissue. Data are shown as means ± SEM of at least five experiments for each group, where statistical significant increase is shown as $*P < 0.05$. $\#P < 0.05$ represents statistical difference *vs* the appropriate parts of the colon of TNBS in CSN-intact rats.

CGRP might promote mucosal integrity by increasing intestinal blood flow.

In conclusion, our results suggest that CSN may play a significant protective/anti-inflammatory role in the colon under physiological and pathological conditions. Finally, involvement of an impairment of the capsaicin-sensitive innervation of the intestine in the development of human IBD (especially Crohn's disease) can not be excluded. Indeed, it has been demonstrated that in patients with Crohn's disease the concentration of SP in rectal dialysates tends to be lower (Bernstein *et al*, 1993) and in these patients the expression of SP receptors was shown to be increased (Mantyh *et al*, 1995).

Acknowledgments

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II.

Szepes Z., Morschl É., Kiss J., Pávó I., Whittle B.J.R., Varga Cs., László F.A., László F.

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Detrimental effects of oestradiol on cysteamine-induced gastroduodenal ulceration in the female rat

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Abstract — The actions of the female sex steroid, oestradiol on cysteamine-induced mucosal ulceration has been evaluated in female Wistar rats. Administration of cysteamine (400 mg·kg^{-1}, s.c.) provoked macroscopic gastroduodenal mucosal injury (assessed planimetrically) and an increase in microvascular permeability (assessed by the extravasation of radiolabeled albumin) in the stomach and duodenum, determined 24 h later. Ovariectomy (2 weeks before cysteamine) reduced gastroduodenal macroscopic injury, and albumin extravasation following cysteamine challenge. Administration of oestradiol (1–5 mg·kg^{-1}, as an i.m. depot 1 week before cysteamine) dose-dependently augmented gastric and duodenal macroscopic mucosal lesions and microvascular permeability provoked by cysteamine. These findings indicate that oestradiol can exacerbate gastroduodenal ulceration and microvascular injury. © 1999 Elsevier Science Ltd. Published by Éditions scientifiques et médicales Elsevier SAS

sex steroids / ovariectomy / oestradiol / mucosal ulceration / micro-vascular permeability / cysteamine

1. Introduction

On the basis of experimental and clinical observations, it is known that ulceration of the gastroduodenal mucosa shows sexual dimorphism. Thus, in the fertile age, peptic ulcer disease occurs more frequently among men than women [2]. In experimental models of mucosal damage, this sexual difference of mucosal injury has also been found. Oral administration of ethanol caused more severe gastric mucosal erosions in male rats than in females [8, 9]. In this model of mucosal damage, gonadectomy protected the stomach against ethanol-induced injury in male rats [8]. Moreover, administration of the testosterone-synthase inhibitor, cyproterone acetate attenuated gastric hemorrhagic erosions in intact male rats following ethanol challenge [8], and reduced gastric lesions and duodenal ulceration provoked by cysteamine [11]. Such findings suggest that endogenous sex hormones might possibly play a role in the development of gastroduodenal mucosal ulceration.

The aim of the present study was to evaluate the actions of the female sex steroid, oestradiol on the generation cysteamine-induced gastroduodenal ulceration in the rat. Thus, in female rats, we examined the effects of ovariectomy and oestradiol on the severity of macroscopically detectable lesions as well as the changes in microvascular permeability in the stomach and duodenum provoked by cysteamine.

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2. Materials and methods

2.1. Experimental protocol

Female Wistar rats (200–220 g) were injected with cysteamine (400 mg·kg^{-1}, s.c.). Twenty-four hours after cysteamine administration, the animals were killed by ether overdose, and their stomach and duodenum were removed. During this 24-h period, rats were deprived of food, but received water ad libitum. For the determination of plasma leakage, ^{125}I-labeled human serum albumin ([^{125}I]-HSA, 2 μCi·kg^{-1}, i.v.) was administered 2 h before autopsy. In some groups of rats, ovariectomy was performed under ether anaesthesia 2 weeks before any experimental procedure. For additional groups of rats, a depot injection of oestradiol (1–5 mg·kg^{-1}, i.m.) were administered 1 week before cysteamine challenge.

2.2 Macroscopic evaluation of lesions

The area of damage and the total mucosal surface of the stomach and duodenum were measured by a digital planimeter (Sokkia Planimeter KP-82-N, Japan), and the injured area was expressed as percentage of the total mucosal surface.

2.3. Albumin leakage

Leakage of [^{125}I]-HSA was determined in the stomach and duodenum as an index of microvascular damage. Blood was collected from the abdominal aorta into syringes containing trisodium citrate (final

concentration 0.318%) and centrifuged (10 000 g, 10 min, 4 °C). The [^{125}I]-HSA content of the plasma and segments of gastric and duodenal tissue was determined in a gamma-spectrometer (Nuclear Enterprises NE 1600) and the albumin content in the stomach and duodenum calculated, taking into account any changes in gastroduodenal blood volume as described previously [4]. Values from control tissues were subtracted from the values of treated tissue and the data were expressed as plasma leakage, μL plasma·g^{-1} wet tissue.

2.4. 17-β-oestradiol plasma level

Plasma 17-β-oetradiol levels were determined after ovariectomy by a radioimmuno-assay method described previously [8].

2.5. Materials

[^{125}I]-labeled human serum albumin was obtained from Izinta (Budapest, Hungary). Oestradiol benzoate was purchased from Intervet International (Boxmeer, the Netherlands). All other compounds were from Sigma Chemical Company.

2.6. Statistical evaluation

The data are expressed as mean ± SE from (n) rats per experimental group. For statistical comparisons, Student's t-test test for unpaired data or analysis of variance with the Bonferroni test were used, where $P < 0.05$ was taken as significant difference.

3. Results

3.1. Gastroduodenal ulceration provoked by cysteamine

Administration of cysteamine (400 mg·kg^{-1}, s.c.) provoked macroscopic injury in the gastric and duodenal mucosa (4.5 ± 0.6% and 12.9 ± 1.0% of total mucosal area, respectively; $n = 7$, $P < 0.001$) as shown in *figure 1*. Cysteamine challenge also increased microvascular permeability in the stomach (Δ57 ± 9 μL·g^{-1} tissue, $n = 7$, $P < 0.001$) and duodenum (Δ155 ± 6 μL·g^{-1} tissue; $n = 7$, $P < 0.001$) after 24 h, as shown in *figure 2*.

3.2. Effects of ovariectomy

Ovariectomy (2 weeks before cysteamine challenge) decreased plasma 17-β-oestradiol level (from 182 ± 10 to 87 ± 5 pmol·L^{-1}, $n = 6$). Ovariectomy significantly lowered the gastric and duodenal macroscopic mucosal injury provoked by cysteamine (by

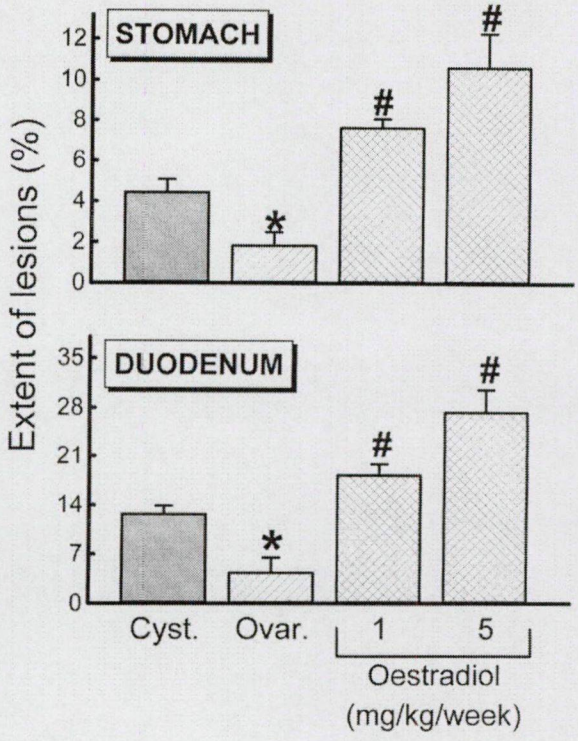

Figure 1. Induction of rat gastric and duodenal mucosal macroscopic injury by cysteamine (Cyst. 400 mg·kg^{-1}, s.c.) after 24 h. The inhibition of cysteamine-induced damage by ovariectomy (Ovar., 2 weeks before cysteamine challenge) and the potentiation of cysteamine-provoked mucosal lesions by oestradiol (1–5 mg·kg^{-1}·week^{-1}, i.m.) pretreatment is shown. The columns show the extent of gastric and duodenal macroscopic lesions (expressed as lesion area, percentage total mucosal area). Data are given as the mean ± SE of 4–7 rats per group; statistical significance is shown as $^{*}P < 0.05$, inhibition of cysteamine-induced lesion; $^{\#}P < 0.05$, potentiation of cysteamine-induced lesion.

62 ± 13% and 65 ± 15%, respectively; $P < 0.001$, $n = 5$) as shown in *figure 1*. Ovariectomy likewise attenuated albumin extravasation in the gastric mucosa and duodenum provoked by cysteamine (by 63 ± 19% and 65 ± 7%, respectively; $P < 0.001$, $n = 5$) as shown in *figure 2*.

3.3. Effects of oestradiol

Administration of oestradiol (1–5 mg·kg^{-1}·week^{-1}, i.m.) to unoperated rats dose-dependently augmented gastric and duodenal macroscopic mucosal lesions induced by cysteamine (by 138 ± 35% and by 114 ± 23%, respectively; $P < 0.001$, $n = 4$). This treatment also increased the microvascular injury in the stomach and duodenum (by 182 ± 59% and by

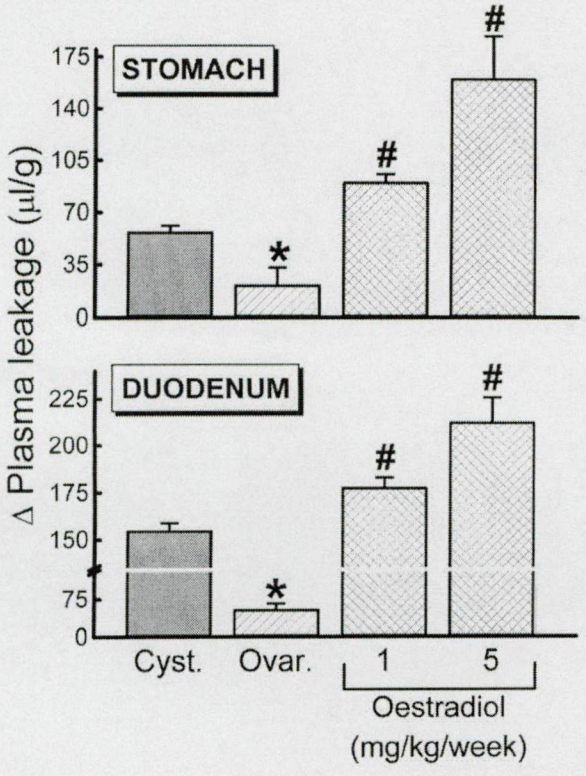

Figure 2. Induction of rat gastric and duodenal vascular leakage of plasma (determined using radiolabeled albumin) by cysteamine (Cyst., 400 mg·kg^{-1}, s.c.) after 24 h. The inhibition of cysteamine-induced plasma leakage by ovariectomy (Ovar., 2 weeks before cysteamine challenge) and potentiation of cysteamine-provoked plasma leakage by oestradiol (1–5 mg·kg^{-1}·week^{-1}, i.m.) pretreatment is shown. The columns show the leakage of plasma (Δ μL·g^{-1} tissue) in gastric and duodenal tissues. Data are given as the mean ± SE of 4–7 rats per group; statistical significance is shown as $^{*}P < 0.05$, inhibition of cysteamine-induced plasma leakage; $^{\#}P < 0.05$, potentiation of cysteamine-induced plasma leakage.

38 ± 5%, respectively; $P < 0.01$, $n = 4$) provoked by cysteamine (*figures 1* and *2*, respectively).

4. Discussion

This study in female rats following ovariectomy extends the previous observations in males that following gonadectomy the gastroduodenal mucosa is less sensitive to various ulcerogenic stimuli [8, 11]. The reduction by ovariectomy of cysteamine-induced ulcers of the stomach and duodenum could be related to the observed decrease of plasma oestradiol levels. Indeed, mucosal damage in response to challenge was increased by exogenous administration of oestradiol into intact female rats.

It is known that an increase of gastric parietal cell mass has a beneficial action against the development of mucosal lesions following ulcerogenic challenge and accelerates ulcer healing [6, 7]. Since ovariectomy enhances gastric parietal cell mass [1], this process may contribute to the mechanism by which ovariectomy protected the mucosa against cysteamine-induced injury. Other factors are also likely to be involved in the apparent aggressive actions of oestradiol towards the gastroduodenal mucosa. Thus administration of oestrogens can lead to the inactivation of prostaglandins in the stomach [5], and prostaglandins are known to have key importance in the defense mechanism of the gastroduodenal mucosa [13, 16].

Here, cysteamine challenge also provoked microvascular plasma leakage, as a measure of vascular endothelial damage. This finding is in agreement with the previous suggestion that aggressive vascular factors are involved in the generation of cysteamine-induced gastroduodenal ulcers [3]. It is well-established that microcirculatory injury and the consequent hypoxia are among those important factors which are considered to lead to gastroduodenal mucosal ulceration [14, 15]. As with the macroscopic injury, in our present work, the reduction in endogenous oestradiol synthesis following ovariectomy lead to the attenuation of cysteamine-induced microvascular leakage. In contrast, augmentation of microvascular leakage followed the administration of exogenous oestradiol after cysteamine challenge. Although further studies are needed to evaluate whether the observed microvascular defects are the consequence or the cause of cysteamine-provoked mucosal injury, it is relevant that oestrogens have vasoconstrictor actions by modulating the pressor effect of vasopressin [12]. The significant aggressive role of endogenous vasopressin via its pressor receptors in the development of gastroduodenal mucosal ulceration has been described previously [10, 12].

In conclusion, oestradiol appears to exert a proulcerogenic action in the generation of macroscopic and microvascular injury in the stomach and duodenum following cysteamine administration in female rats. Thus inappropriate levels of endogenous oestradiol might possibly be involved in the pathogenesis of gastroduodenal mucosal damage.

Acknowledgments

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III.

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Cytotoxicity associated with induction of nitric oxide synthase in rat duodenal epithelial cells *in vivo* by lipopolysaccharide of *Helicobacter pylori*: inhibition by superoxide dismutase

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1 The products released by *Helicobacter pylori* (*H. pylori*) in the gastric antral and duodenal mucosa may be involved in mucosal ulceration by stimulating the local formation of cytotoxic factors such as nitric oxide (NO), superoxide or peroxynitrite.

2 The present study investigates the ability of purified *H. pylori* lipopolysaccharide (LPS) to induce nitric oxide synthase (iNOS) in rat duodenal epithelial cells following *in vivo* challenge and its interaction with superoxide in promoting cellular damage and apoptosis.

3 *H. pylori* LPS (0.75–3 mg kg^{-1} i.v. or 3–12 mg kg^{-1} p.o.) induced a dose-dependent expression of iNOS activity after 5 h in the duodenal epithelial cells, determined by [^{14}C] arginine conversion to citrulline.

4 The epithelial cell viability, as assessed by Trypan Blue exclusion and MTT conversion, was reduced 5 h after challenge with *H. pylori* LPS, while the incidence of apoptosis was increased.

5 The iNOS activity and reduction in cell viability following *H. pylori* LPS challenge i.v. was inhibited by the selective iNOS inhibitor, 1400 W (0.2–5 mg kg^{-1} i.v.).

6 Concurrent administration of superoxide dismutase conjugated with polyethylene glycol (250–500 i.u. kg^{-1}, i.v.), which did not modify the cellular iNOS activity, reduced the epithelial cell damage provoked by i.v. *H. pylori* LPS, and abolished the increased incidence of apoptosis.

7 These results suggest that expression of iNOS following challenge with *H. pylori* LPS provokes duodenal epithelial cell injury and apoptosis by a process involving superoxide, implicating peroxynitrite involvement. These events may contribute to the pathogenic mechanisms of *H. pylori* in promoting peptic ulcer disease.

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Keywords: *Helicobacter pylori*; nitric oxide; inducible nitric oxide synthase; duodenal epithelial cells; duodenum; lipopolysaccharide

Abbreviations: 1400 W, N-(3-(aminomethyl)benzyl)acetamidine; cNOS, constitutive isoforms of NO synthase; DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazipine-*N*′-2-ethanesulfonic acid; HES, hematoxylin-eosin-safran; iNOS, inducible isoform of NO synthase; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; SOD–PEG, conjugate of polyethylene glycol and superoxide dismutase

Introduction

Infection with *Helicobacter pylori* (*H. pylori*) is a dominant pathogenic factor in peptic ulcer disease (Blaser, 1990). This bacterium colonises the gastric antrum and sites of gastric metaplasia in the duodenum, and induces local inflammation (Carrick *et al.*, 1989). *H. pylori* infection may provoke damage in the stomach and duodenum by releasing soluble factors that activate inflammatory cells such as neutrophils, to produce cytotoxic mediators such as superoxide (Mooney *et al.*, 1991) and nitric oxide (NO) (McCall *et al.*, 1989). High concentration of NO are known to be cytotoxic, and in combination with the superoxide radical, leads to the subsequent formation of the moieties, peroxynitrite and hydroxyl radicals, which are highly injurious to cells (Ischiropoulos *et al.*, 1995; Beckman *et al.*, 1990).

The inducible isoform of NO synthase (iNOS) is capable of the sustained production of high levels of NO (Knowles *et al.*, 1990; Salter *et al.*, 1991). This isoform can be expressed following challenge with endotoxin lipopolysaccharide (LPS), not only in inflammatory cells, but also in gastro-intestinal epithelial cells and its expression is associated with cytotoxicity (Brown *et al.*, 1994; Tepperman *et al.*, 1993; 1994). Since *H. pylori* can synthesize an endotoxin (Moran, 1996), expression of iNOS in gastro-duodenal epithelial cells could play a role in the pathogenesis of mucosal lesions related to infection by this organism. Studies on gastric mucosal biopsies from patients with gastritis associated with *H. pylori* infection exhibited increased antral mRNA for iNOS, as well as iNOS protein in epithelium, endothelium and inflammatory cells, compared with tissue from *H. pylori*–negative gastritis or controls (Fu *et al.*, 1999). In another report, a correlation was found between the iNOS immunostaining, the degree of inflammation, the apoptotic index and the density of *H. pylori* infection, all of

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which decreased on eradication of the bacterium (Hahm *et al.*, 1997).

Previous studies have shown that intravenous challenge with a water extract of *H. pylori* can express iNOS and lead to epithelial injury in the rat duodenum (Lamarque *et al.*, 1998). These effects were inhibited by polymixin B, which binds LPS (Morrison & Jacobs, 1976), suggesting an important role for an LPS in this process. However, although *in vitro* studies have shown that *H. pylori* LPS can lead to the expression of iNOS in murine and human macrophage cell lines in culture, this LPS was only weakly active under those conditions (Perez-Perez *et al.*, 1995). Such findings cast some doubt on the possibility that the expression of iNOS through the actions of the LPS is involved in the pathogenic processes associated with *H. pylori* infection, although *in vitro* studies of that nature do have limitations, including lack of cross-talk between different cell types and mediators. The aim of the present study was, therefore, to investigate the ability of a purified preparation of LPS from *H. pylori* to induce iNOS in duodenal epithelial cells and determine its association with cell damage and apoptosis following its administration *in vivo* to the rat. As the main objective was to evaluate the potential of the LPS to induce iNOS activity in an experimental setting *in vivo*, rather than provide a model of clinical infection, the intravenous route was utilized in the majority of the experiments. However, the ability of this LPS to induce iNOS activity and produce cellular injury after its intragastric instillation was also studied.

To evaluate the role of iNOS in the cytotoxic process, the effects of a highly selective inhibitor of iNOS, 1400 W (N-(3-(aminomethyl)benzyl)acetamidine; Garvey *et al.*, 1997; Laszlo & Whittle, 1997) on epithelial cell injury were evaluated. In addition, to explore further the mechanisms underlying such cellular injury, the involvement of the superoxide, and hence peroxynitrite, on epithelial cell injury provoked by the *H. pylori* LPS was investigated. The effects of a conjugate of superoxide dismutase (SOD–PEG), which has previously been shown to reduce the mucosal injury provoked by local infusion of NO donors in the rat gastric mucosa (Lamarque & Whittle, 1995) was therefore evaluated on the cellular damage and increased apoptosis provoked by the LPS from *H. pylori*.

Methods

Preparation of LPS

Biomass of *H. pylori* (NCTC 11637 strain) was grown in brain–heart infusion containing 2% foetal calf serum to ensure expression of high molecular weight LPS (Walsh & Moran, 1997) Extraction of LPS was performed using a phenol-water procedure (Westphal *et al.*, 1952). Subsequently, extracted LPS was purified by treatment with RNase A, DNase II and proteinase K, and by ultracentrifugation at 100,000 × *g* at 4°C for 18 h (Moran *et al.*, 1992). For suspension, purified LPS was dispersed in endotoxin-free water by sonication.

Animal preparation

Male Wistar rats, weighing 200–250 g, were fasted overnight but allowed free access to water. In the majority of the experiments, purified LPS from *H. pylori* (0.75–3 mg kg^{-1}) was administered *via* a tail vein under transient anaesthesia induced by ether. In control experiments, rats were pretreated with saline (0.5 ml kg^{-1}, i.v.).

In a further series of experiments to evaluate the ability of the LPS to induce iNOS after oral challenge, *H. pylori* LPS (3–12 mg kg^{-1}) dissolved in saline (1.0 ml), was administered intragastrically through a smooth rubber feeding tube.

Duodenal epithelial cell isolation

Duodenal epithelial cells were isolated as described previously (Lamarque *et al.*, 1998; Lentze *et al.*, 1985). A 5 cm segment of duodenum was slowly flushed with 50 ml of a solution containing 0.15 M NaCl and 0.1 mM dithiothreitol (DTT). The segment was then filled with 5 ml of a solution containing (in mM): KCl 1.5, NaCl 96, sodium citrate 27, KH_2PO_4 8 and Na_2HPO_4 5.6 (pH 7.3), and the proximal and the distal ends were ligated. The segment was then immersed in phosphate-buffered saline (PBS) kept at 37°C, which was bubbled with 95% O_2–5% CO_2. After 15 min, the instilled solution was removed and another solution containing 1.5 mM EDTA and 0.5 mM DTT was instilled over 5 min, as described previously (Tepperman *et al.*, 1993). The epithelial cells were collected in suspension in this solution. The cells were washed twice with PBS (pH 7.4) and centrifuged for 5 min at 800 × *g*. The cells were suspended in a buffer containing *N*-2-hydroxyethylpiperazipine-*N'*-2-ethanesulphonic acid (HEPES) (10 mM), sucrose (320 mM), DTT (1 mM), soybean trypsin inhibitor (10 $\mu g\ ml^{-1}$), leupeptin (10 $\mu g\ ml^{-1}$), aprotonin (2 $\mu g\ ml^{-1}$).

To assess the purity of epithelial cells in the aliquots isolated from the duodenum, in some experiments the cells were fixed with formaldehyde, stained by hematoxylin-eosin-safran and counted under light microscopy and expressed as the percentage of epithelial cells by fields.

NO synthase activity

NO synthase activity in duodenal epithelial cells was measured as the conversion of L-[^{14}C]-arginine monohydrochloride to [^{14}C]-citrulline, based on the method described previously (Knowles *et al.*, 1990; Lamarque *et al.*, 1998). Cells were homogenized (30 s, Ultra-Turrax; 5 mm blade) in buffer (pH 7.4) containing HEPES (10 mM), sucrose (32 mM), DTT (1 mM), leupeptin (10 $\mu g\ ml^{-1}$), soybean trypsin inhibitor (10 $\mu g\ ml^{-1}$) and aprotonin (2 $\mu g\ ml^{-1}$).

Following centrifugation (10,000 × *g* 4°C), an aliquot of the supernatant (40 μl) was used for the determination of the enzymatic activity and the remaining kept for protein content measurement by a modification of Bradford's method (Lamarque *et al.*, 1998). The aliquot was placed in 100 μl of the pre-warmed incubation buffer containing (final concentration) potassium phosphate (50 mM; pH 7.4), L-valine (50 mM), $MgCl_2$ (1 mM), $CaCl_2$ (200 μM), DL-dithiothreitol (1 mM), L-citrulline (1 mM), NADPH (0.3 mM), FAD (3 μM), FMN (3 μM), BH_4 (3 μM) L-[^{14}C]arginine monohydrochloride (15.5 nM) and incubated for 10 min at 37°C. The incubation was terminated by binding arginine following the addition of 500 μl of 1:1 suspension of Dowex (AG 50W-8) in water. The resin was allowed to settle (30 min) and 975 μl of supernatant taken for scintillation counting in 3 ml of scintillation liquid.

Product formation that was inhibited by *in vitro* incubation with the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA; 300 μM), but not by ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; 1 mM), was taken as an index of iNOS activity (Salter *et al.*, 1991). The constitutive NOS activity (designated cNOS as the nature of the isoform was not established) was taken as that activity inhibited by both L-NMMA and EGTA.

Duodenal epithelial cells viability

The viability of duodenal epithelial cells was determined in cells collected from rats that had been challenged, 5 h previously, with purified *H. pylori* LPS or saline. The viability of cells was determined by Trypan blue dye exclusion (0.5%, Trypan blue in PBS) as described previously (Tepperman *et al.*, 1991). The number of viable cells was determined by light microscopy (×40 magnification) by counting those cells that excluded the dye. Cells were counted in a randomized manner using a haemocytometer.

Viability of duodenal epithelial cells was determined also by the conversion of the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to the formazan salt by mitochondrial dehydrogenases (Mosmann, 1983). Briefly, 300 μl of MTT was added to 50 μl of suspension of the homogenized cells prepared as described above and suspended in a buffer containing HEPES (10 mM), sucrose (320 mM), DTT (1 mM), soybean trypsin inhibitor (10 μg ml^{-1}), leupeptin (10 μg ml^{-1}), aprotonin (2 μg ml^{-1}). After 4 h of incubation at 37°C, the suspension was centrifuged (10,000 × *g*, 2 min), the pellet was solubilized in DMSO (1 ml) and centrifuged again. The spectrophotometric absorbance of the formazan salt was measured in the supernatant at 540 nm. Protein content in the initial suspension of homogenized cells was determined as above. Results were expressed as OD mg^{-1} of protein.

Effects of H. pylori *LPS on NO synthase activity and viability in duodenal epithelial cells*

At 5 h after administration of *H. pylori* LPS (0.75–3 mg kg^{-1} i.v or 3–12 mg kg^{-1} p.o.), the animals were killed by cervical dislocation. The duodenum was removed, and duodenal epithelial cells isolated for the determination of iNOS activity and cell viability. At this time after LPS (3 mg kg^{-1}, i.v.) challenge, preliminary histological evaluation of the duodenal tissue indicated some areas of epithelial injury.

In further experiments, rats were treated with the selective iNOS inhibitor, 1400 W (0.2–5 mg kg^{-1} i.v.) or saline, concurrently administered with *H. pylori* LPS (3 mg kg^{-1}, i.v.). The dose of 1400 W was taken from previous *in vivo* studies on rat gastrointestinal tissue (Laszlo & Whittle, 1997).

In a separate series of studies, the activity of *H. pylori* LPS (3 mg kg^{-1}) on iNOS induction and cell viability was compared with that of *E. coli* LPS (3 mg kg^{-1}), 5 h after intravenous administration.

In a further group of rats, a systemically acting conjugate of polyethylene glycol and superoxide dismutase (SOD–PEG; 250–500 i.u. kg^{-1}) or isotonic saline was administered by an intravenous bolus injection, 15 min prior to *H. pylori* LPS administration (3 mg kg^{-1}, i.v.). The doses of SOD–PEG were taken from previous studies on its inhibitory action on the inflammatory response in the rat skin following systemic administration (Boughton-Smith *et al.*, 1993) and its action in preventing gastric mucosal injury induced by local intra-arterial infusion of NO donors (Lamarque & Whittle, 1995). The viability, and iNOS activity was determined in duodenal cells from rats treated by SOD–PEG or saline, 5 h after *H. pylori* LPS administration.

Determination of apoptosis

The degree of apoptosis was determined in duodenal epithelial cells collected from rats that had been challenged, 5 h previously, with purified *H. pylori* LPS (3 mg kg^{-1}, i.v.) or saline ($n=5$ for each). The cells were fixed with buffered formaldehyde for 10 min and then incubated with 1 mg ml^{-1} 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) for 15 min at 37°C. Cells were evaluated by fluorescent microscopy, and nuclei with highly condensed and fragmented chromatin were considered apoptotic. The percentage of apoptotic cells was determined in 15 different fields per preparation of duodenal cells, with 40 cells per field being evaluated.

In a further group ($n=5$), the effect of pretreatment with SOD–PEG (500 i.u. kg^{-1} i.v.) on the degree of apoptosis was also evaluated.

Materials

All chemical compounds were obtained from Sigma Chemical Co (Sigma France, St Quentin Fallavier) excepted L-[^{14}C]arginine monohydrochloride obtained from Amersham France (Les Ulis), the scintillation liquid Ready safe from Beckman (Division Biorecherche, Paris Nord II, Villepinte, France) and 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) from Boehringer, Mannheim, Germany. The SOD–PEG was obtained from Oxis international Inc (NewYork, U.S.A.). 1400 W (N-(3-(aminomethyl)benzyl)acetamidine) was provided as a gift from GlaxoWellcome (Stevenage, U.K).

Statistical analysis

The data are expressed as the mean±s.e.m. of (n) rats per group. Statistical comparisons were made by analysis of variance with the Bonferroni test where $P<0.05$ was taken as significant.

Results

Induction of iNOS in duodenal epithelial cells after H. pylori *LPS*

No significant increase in the low basal iNOS activity, determined as that NOS activity which was inhibited by L-NMMA but not by *in vitro* incubation with EGTA (1 mM), could be detected in the supernatants of the lysed duodenal epithelial cells obtained from animals challenged with saline alone. Following i.v. administration of LPS (0.75–3 mg kg^{-1}), a dose-dependent increase in iNOS activity was detected, determined after 5 h, as shown in Figure 1. This iNOS activity remained at a similar level when determined 7 h after challenge. The basal cNOS activity (705±152 pmol min^{-1} mg protein^{-1}; $n=10$) did not change 5 h after challenge with 3 mg kg^{-1} of LPS (728±172 pmol min^{-1} mg protein^{-1}; $n=10$).

A significant dose-dependent increase in iNOS activity was also detected 5 h after intragastric administration of LPS, reaching 78±56, 136±82 and 244±82 pmol min^{-1} mg protein^{-1} ($P<0.05$ for each; $n=8$) following the dose of 3, 6 and 12 mg kg^{-1} respectively.

Effect of H. pylori *LPS on duodenal epithelial cell viability*

The proportion of epithelial cells in the cell suspension isolated from the duodenum by dispersion was 98±2% ($n=4$), as determined by microscopy, the other cells identified by morphological analysis being mastocytes.

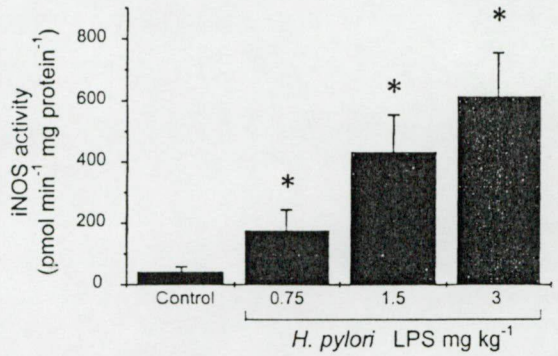

Figure 1 Dose-dependent increase in inducible nitric oxide (iNOS) activity in isolated duodenal epithelial cells, harvested 5 h following challenge with *H. pylori* lipopolysaccharide (LPS; 0.75–3 mg kg^{-1}, i.v.) in rat. Data, shown as the iNOS activity (pmol min^{-1} mg $protein^{-1}$), are mean±s.e.mean of 5–10 experiments, where * denotes a significant difference from the control group ($P<0.05$).

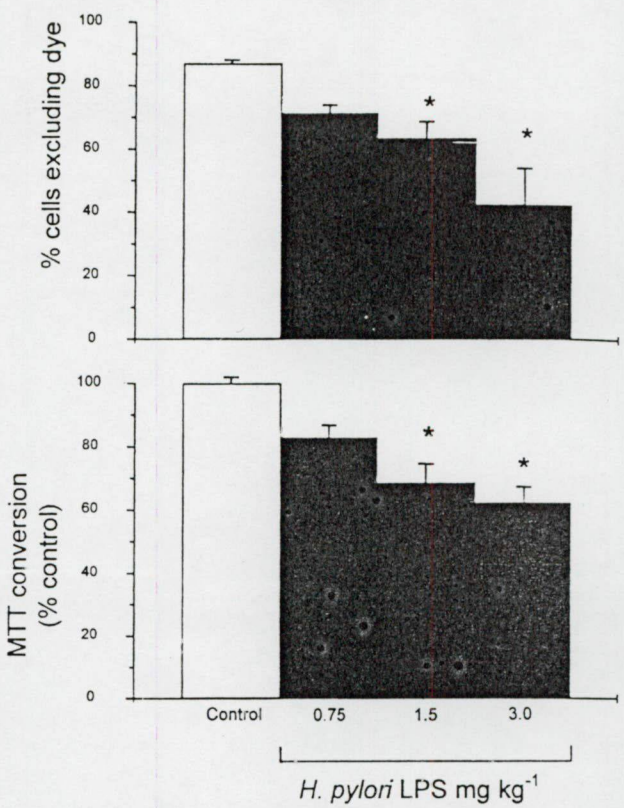

Figure 2 Percentage of viable isolated duodenal epithelial cells, as assessed by Trypan blue exclusion (per cent cells excluding dye: upper graph) or MTT conversion (per cent control; lower graph), 5 h following challenge with *H. pylori* lipopolysaccharide (LPS: 0.75–3 mg kg^{-1}, i.v.). Data are mean±s.e.mean of 5–12 experiments where * denotes a significant difference from the saline control group ($P<0.01$).

The proportion of non-viable intestinal cells isolated from control rats, assessed by Trypan Blue staining, was 13±1%; $n=12$. The intravenous administration of *H. pylori* LPS (0.75–3 mg kg^{-1}) provoked a significant dose-dependent increase of the number of non-viable cells when assessed by dye exclusion 5 h later (Figure 2). Likewise, following *H. pylori* LPS (0.75–3 mg kg^{-1}, i.v.) administration, the percentage of non-viable cells, estimated by MTT conversion, was dose-dependently increased, as shown in Figure 2.

Following intragastric administration of *H. pylori* LPS (3, 6 and 12 mg kg^{-1}), the proportion of non-viable intestinal cells as assessed by Trypan blue staining, was 12±1, 15±1 and 20±1% ($n=12$) respectively, being significantly greater ($P<0.05$) than the control at doses of 6 and 12 mg kg^{-1}.

Effects of 1400 W

The increase in iNOS activity, determined in the epithelial cells 5 h after intravenous challenge with *H. pylori* LPS, was inhibited dose-dependently by concomitant administration of 1400 W (0.2–5 mg kg^{-1}, i.v.) as shown in Figure 3. The cNOS activity measured 5 h after LPS challenge was not significantly modified by the treatment with 1400 W (5 mg kg^{-1}, i.v.), being 643±234 and 684±176 pmol min^{-1} mg $protein^{-1}$ ($n=8$) respectively.

The increase in non-viable cells, estimated by Trypan blue dye exclusion or MTT conversion 5 h after the LPS injection, was dose-dependently reduced by concomitant treatment of the rats with 1400 W (0.2–5 mg kg^{-1}, i.v.), as shown in Figure 4.

Comparison of the activity of H. pylori *LPS and* E. coli *LPS on cellular iNOS and damage*

In a single-dose comparative study, the increase in iNOS activity in duodenal epithelial cells was Δ 224±34 pmol min^{-1} mg $protein^{-1}$ ($n=8$) 5 h following *H. pylori* LPS administration (3 mg kg^{-1} i.v.), whereas that observed after *E. coli* LPS administration (3 mg kg^{-1} i.v.) was Δ 463±27 pmol min^{-1} mg $protein^{-1}$ ($n=8$).

The proportion of non-viable cells, estimated by Trypan blue staining, that had been isolated from rats challenged with *H. pylori* LPS (27±5%, $n=6$) was not significantly different than that of *E. coli* LPS-challenged rats (25±4%, $n=6$). Likewise, a comparable injurious effect was also found in both groups when the percentage of non-viable cells was assessed by MTT conversion (38±3%, $n=6$, and 38±4%, $n=6$, respectively).

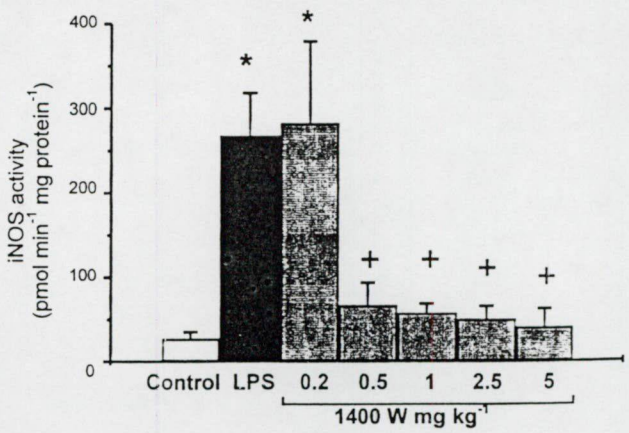

Figure 3 Inducible nitric oxide synthase (iNOS) activity in duodenal epithelial cells 5 h following challenge with *H. pylori* lipopolysaccharide (LPS; 3 ml kg^{-1}, i.v.) in rat treated concurrently with saline (control) or with N-(3-(aminomethyl)benzyl)acetamidine (1400 W; 0.2–5 mg kg^{-1}, i.v.). Data, shown as iNOS activity (pmol min^{-1} mg $protein^{-1}$), are mean±s.e.mean of 5–9 experiments, where * denotes a significant difference from the control ($P<0.01$) and + a significant difference from the LPS alone group ($P<0.01$).

Effects of SOD–PEG

Administration of SOD–PEG (500 i.u. kg^{-1}, i.v.), 15 min prior to challenge with *H. pylori* LPS, did not significantly

affect the increase in iNOS activity in duodenal epithelial cells, determined 5 h after challenge (Figure 5). The cNOS activity measured 5 h after *H. pylori* LPS challenge was likewise not affected following SOD–PEG administration (652±228 and 796±175 pmol min^{-1} mg $protein^{-1}$, $n=10$, respectively).

The proportion of non-viable intestinal cells, assessed by Trypan blue staining, that was isolated from rats treated 5 h previously with SOD–PEG (500 i.u. kg^{-1}, i.v.) alone, was not different from those of cells taken from control rats (12±1%; $n=6$ compared with 12±2%, $n=5$). However, the increase in non-viable cells 5 h after *H. pylori* LPS administration was prevented by pretreatment of the rats with SOD–PEG (250–500 i.u. kg^{-1}, i.v.), 15 min prior to challenge (Figure 6). Likewise, the increase in non-viable cells assessed by MTT conversion after *H. pylori* LPS challenge was prevented by pretreatment with these doses of SOD–PEG (Figure 6).

Effects of H. pylori *LPS and SOD–PEG on apoptosis*

The percentage of apoptotic epithelial cells, assessed by the condensed chromatin fragments in the nuclei and by the segmentation of the nuclei after DNA staining, 5 h following *H. pylori* LPS injection, was significantly increased to 11.0±0.6% ($n=5$; $P<0.05$) as compared to the control group (5.3±0.8%; $n=5$). This increase in the incidence of apoptosis in the cells following LPS challenge was prevented by the pretreatment with SOD–PEG (500 i.u. kg^{-1}, i.v.), 15 min prior to challenge (5.6±0.4%; $n=5$; $P<0.05$ compared with LPS alone).

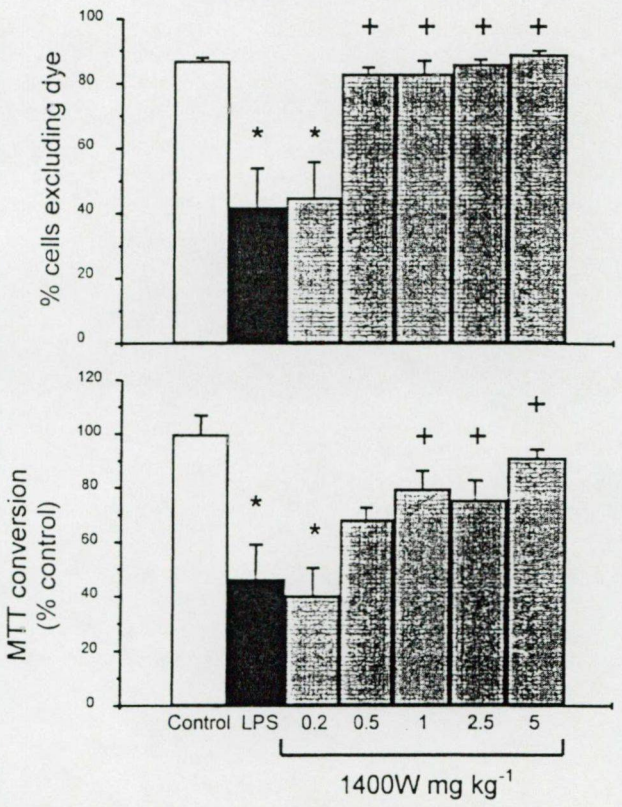

Figure 4 Percentage of viable isolated duodenal epithelial cells, as assessed by Trypan blue exclusion (per cent cells excluding dye; upper graph), or MTT conversion (per cent control; lower graph), 5 h following challenge with *H. pylori* lipopolysaccharide (LPS; 3 ml kg^{-1}, i.v.) in rat treated concurrently with saline (control) or with N-(3-(aminomethyl)benzyl)acetamidine (1400 W; 0.2–5 mg kg^{-1}, i.v.). Data are mean±s.e.mean of 5–9 experiments, where * denotes a significant difference from the control ($P<0.01$) and + a significant difference from the LPS alone group ($P<0.05$).

Discussion

In a previous study, iNOS activity was detected in rat duodenal tissue, as well as in isolated duodenal epithelial cells after the

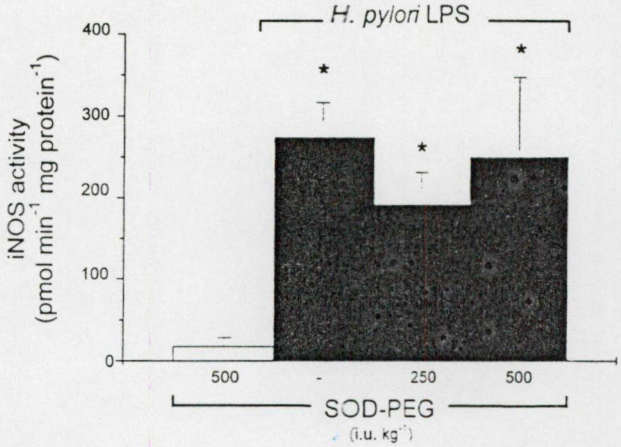

Figure 5 Inducible nitric oxide (iNOS) activity in duodenal epithelial cells, 5 h following challenge with *H. pylori* lipopolysaccharide (LPS; 3 mg kg^{-1}, i.v.) in rat treated concurrently with saline or with superoxide dismutase conjugated with polyethylene glycol (SOD–PEG; 250–500 i.u. kg^{-1}, i.v.). Data, expressed as iNOS activity (pmol min^{-1} mg $protein^{-1}$) are mean±s.e.mean of 5–10 experiments, where * denotes a significant difference from the control ($P<0.01$). In control experiments, a group of rats received SOD–PEG (500 i.u. kg^{-1}, i.v.). There was no significant difference ($P>0.05$) between values for LPS alone and LPS with either dose of SOD–PEG.

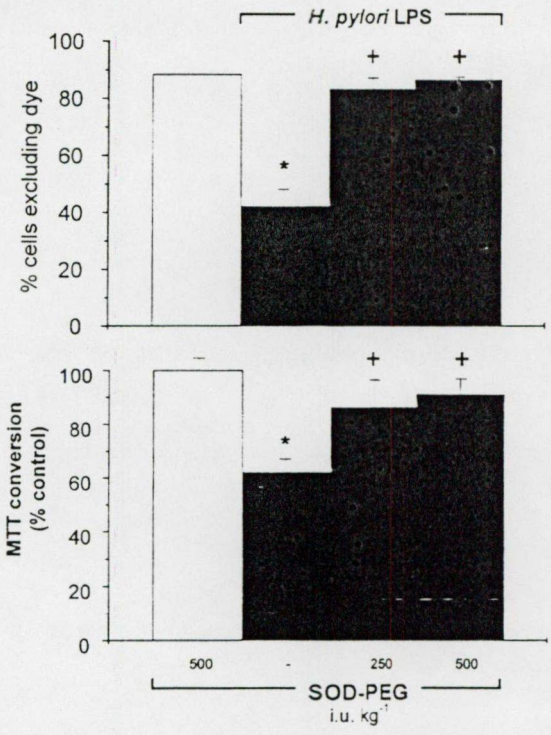

Figure 6 Percentage of viable isolated duodenal epithelial cells, as assessed by Trypan blue exclusion (per cent of cells excluding dye; upper graph), or MTT conversion (per cent control; lower graph), 5 h following challenge with *H. pylori* lipopolysaccharide (LPS; 3 mg kg^{-1}, i.v.) in rat treated concurrently with saline or with superoxide dismutase conjugated with polyethylene glycol (SOD–PEG; 250–500 i.u. kg^{-1}, i.v.). In control experiments, a group of rats received SOD–PEG (500 i.u. kg^{-1}, i.v.). Data are mean± s.e.mean of 5–10 experiments, where * denotes a significant difference from the control ($P<0.05$) and + denotes a significant difference from the *H. pylori* LPS group alone ($P<0.05$).

intravenous administration of an *H. pylori* water extract (Lamarque *et al.*, 1998). In the present investigation, a dose-dependent elevation of iNOS activity was observed in rat duodenal epithelial cells 5 h after intravenous challenge with a purified LPS from *H. pylori*. In addition, dose-dependent iNOS induction following a single intragastric administration of *H. pylori* LPS was likewise observed in the current study. The higher doses required by this latter route may reflect the requirement of penetration of the LPS through the mucus barrier overlying the epithelium.

Expression of iNOS in colonic and small intestinal epithelial cells following *E. coli* endotoxin challenge is associated with a reduction in epithelial cell viability (Tepperman *et al.*, 1993; 1994). Likewise, in the present study, a reduction in duodenal epithelial cell viability, determined *ex vivo* using both Trypan blue dye exclusion and the MTT mitochondrial assay on harvested cells, was observed 5 h after intravenous administration of the *H. pylori* LPS. An increase in the incidence of apoptosis was also observed in these duodenal cells following challenge with this LPS. Duodenal epithelial cell injury was likewise observed after the single intragastric administration of the LPS. Recent studies have also shown that the repeated intragastric administration of an *H. pylori* LPS preparation, that induced iNOS in the rat gastric mucosa, could also provoke apoptosis in the gastric epithelial cells (Slomiany *et al.*, 1998a,b).

Other studies have demonstrated that duodenal perfusion of an extract of *H. pylori* reduced alkaline secretion in the rat, considered to reflect inhibition of NO formation by agents formed from local peptidase activity on proteins contained in the crude extract (Fandriks *et al.*, 1997). Comparable effects on alkaline secretion were not seen with extracts of *E. coli*. However, any such inhibitors would be unlikely to influence the present results obtained following parenteral administration of purified LPS.

Our results suggest that the activity of *H. pylori* LPS on iNOS expression, as well as cytotoxicity, in duodenal epithelial cells were of a similar order of magnitude to that observed with *E. coli*, at the dose investigated, although dose-response studies were not conducted. Such a finding *in vivo* is in contrast to previous studies with LPS *in vitro* (Perez-Perez *et al.*, 1995; Shapiro & Hotchkiss, 1996). Thus, although *H. pylori* LPS induced the production of NO from macrophages in culture, it was 2×10^4 fold less potent than the LPS from *E. coli*, which may reflect both the nature of the *in vitro* study and the bone-marrow derived cell type utilized for iNOS expression in those studies.

It has been established that administration *in vivo* of preparations of *H. pylori* LPS can provoke the release of pro-inflammatory cytokines, which may also be involved in the pathological processes (Slomiany *et al.*, 1998b). These cytokines released by *H. pylori* may also act through NO-dependent pathways (Crabtree, 1998) since they are potent inducers of the iNOS enzyme. It is feasible that the initiation of a cascade of pro-inflammatory mediators *in vivo* that can induce iNOS, may in part, explain the activity seen under these present conditions compared with the low potency of the *H. pylori* LPS for iNOS expression *in vitro*, confirming the importance of *in vivo* models in the understanding of these pathological processes.

In the present study, the reduction in cell viability and the iNOS activity that followed challenge with the *H. pylori* LPS was inhibited by concurrent administration of 1400 W, known to be a highly selective inhibitor of iNOS (Garvey *et al*, 1997; Laszlo & Whittle, 1997). Indeed, at the doses of 1400 W that abolished iNOS activity, no greater cellular injury than under control conditions was detected by dye-exclusion or the MTT assay. These findings strongly implicate the involvement of the iNOS activity in the process that lead to the epithelial cell injury following challenge with the LPS. The data also suggest that the levels of iNOS activity, rather than the total NO synthase activity, is of importance in the process of cell injury. This may reflect the prevailing conditions of the microenvironment which lead to iNOS expression, especially the presence of other cytotoxic moieties and the subsequent interactions of the NO so generated.

Intravenous administration of the systemically active conjugate, SOD–PEG, that scavenges superoxide, did not prevent the expression of iNOS activity, but did inhibit the cell damage induced by *H. pylori* LPS. Such findings, therefore, support the involvement of the superoxide anion, along with NO, in the cell damage induced by *H. pylori* LPS. These findings also imply that a close correlation between only one potential cytotoxic process, such as iNOS expression alone, would not be anticipated, especially if these processes interact synergistically. In recent studies, the damage induced by intravenous challenge with *E. coli* LPS in rat small intestinal epithelial cells, using similar techniques as described previously (Lamarque *et al.*, 1998), has also been shown to be attenuated by administration of a SOD-mimetic (Salvamini *et al.*, 1999).

Sonicates of *H. pylori* have been shown to induce an oxidative burst in human polymorphonuclear and monocytes (Nielsen & Andersen, 1992), and purified LPS has been shown to prime neutrophils for increased activity on subsequent stimulation (Nielsen *et al.*, 1994). In addition, an increased luminol chemiluminesence, which reflects the generation of reactive oxygen species, has been found in the gastric antrum of patients infected by *H. pylori* (Davies *et al.*, 1994). The local release of these cytotoxic oxygen radicals has hence been suggested to play a role in the mucosal lesions observed in peptic ulcer disease associated with *H. pylori* (Nielsen & Andersen, 1992; Davies *et al.*, 1994). Increased iNOS expression has also be observed in gastric biopsies from patients with *H. pylori*-associated gastritis (Hahm *et al.*, 1997; Fu *et al.*, 1999). By interacting with NO formed by iNOS, these oxygen species may form further damaging products such as peroxynitrite that can induce lipid peroxidation (Beckman *et al.*, 1990). Such reactive species may also provoke epithelial cell injury by activating poly (ADP-ribose) synthase that depletes the intracellular energy store (Kennedy *et al.*, 1998). Although superoxide production has not determined in the present study using freshly isolated duodenal epithelial cells, it has been demonstrated in previous studies in rat gastric epithelial cells in culture following ethanol challenge (Hirashi *et al.*, 1999). Moreover, the damage induced by intravenous challenge with *E. coli* LPS in rat small intestinal epithelial cells has been shown to be reduced by agents that act as peroxynitrite decomposition catalysts (Salvemini *et al.*, 1999), providing support to the involvement of peroxynitrite in such cytotoxicity.

The increase in DNA fragmentation, as an index of apoptosis, observed in duodenal epithelial cells from rats challenged with *H. pylori* LPS in the present study, was suppressed in cells from rats pretreated with SOD–PEG. These results suggest the involvement of peroxynitrite in the NO-dependent apoptopic process. The mechanism of the apoptosis induced by peroxynitrite in neuronal cells is considered to involve the Bcl-2 pathway and the impairment of the mitochondrial function (Almeida *et al.*, 1998; Keller *et al.*, 1998). It is therefore relevant that mitochondrial function assessed by MTT conversion was diminished after *H. pylori* LPS challenge which was prevented SOD–PEG pretreatment,

suggesting that this cytotoxic process also operates in the duodenal epithelial cells.

The expression of iNOS in duodenal epithelial cells could reflect a host–defence mechanism against colonization by *H. pylori*, since NO can exert bactericidal actions (Evans *et al.*, 1996; Granger *et al.*, 1988). Furthermore, the present findings indicate that induction of iNOS can provoke local epithelial cytotoxicity as well as stimulating apoptosis, which would thus lead to the clearance of the epithelial cells on which *H. pylori* was adhering (Kim *et al.*, 1998).

The current findings thus give support to the concept that release of *H. pylori* LPS *in vivo* may lead to the local production of elevated concentrations of NO from duodenal epithelium and possibly other mucosal cells, through the expression of iNOS. The findings that the cytotoxic actions and apoptosis in these cells following challenge with *H. pylori* LPS can be attenuated both by the selective iNOS inhibitor, 1400 W, and by SOD–PEG supports an interactive role of NO and superoxide. Either radical may act independently to cause injury, interacting in a synergistic manner, or may combine to form the reactive species, peroxynitrite, which may underlie them in the cellular injury. If such mechanisms play a role in the pathogenesis of peptic ulceration associated with *H. pylori* infection, or the subsequent development of mucosal atrophy and increased cancer risk (Hahm *et al.*, 1997; Fu *et al.*, 1999; Tatemichi *et al.*, 1998), pharmacological intervention to attenuate the production of these reactive moieties may be of therapeutic benefit.

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Attenuation of *Helicobacter pylori* endotoxin-provoked rat intestinal inflammation by selective inhibition of the inducible nitric oxide synthase

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Abstract

We studied the actions of purified *Helicobacter pylori* endotoxin (3 mg kg^{-1}, i.v.) on rat intestinal vascular permeability (assessed by the radiolabelled human serum albumin leakage technique) and on nitric oxide synthase induction (assessed by the citrulline assay) 4 h later. We found increased albumin leakage and expression of the inducible nitric oxide synthase in jejunum and colon, effects reversed by a selective inducible nitric oxide synthase inhibitor N-(8-(aminomethyl)benzyl)-acetamidine (1400W; 0.2–1 mg kg^{-1}, s.c., concurrently with endotoxin). Thus, *H. pylori* endotoxin seems to be capable of provoking an inflammatory response in the rat intestinal tissue. Systemic liberation of *H. pylori* endotoxin might possibly attenuate jejunal and colonic mucosal barrier function, a process mediated by the expression of the inducible nitric oxide synthase.

Keywords: *Helicobacter pylori* endotoxin; Intestinal inflammation; Inducible nitric oxide synthase

1. Introduction

Helicobacter pylori is an acid-resistant bacterium, which can grow only at low pH, predominantly in the antral part of the stomach. The incidence of *H. pylori* infection is high, approaching 50–80% in European adults. The pathological involvement of *H. pylori* is known in gastroduodenal inflammation and duodenal ulceration, and it might participate in the generation of gastric malignancies [6]. Although the clinical impact of a better understanding of how *H. pylori* affects gastrointestinal mucosa is high, only few investigations had been carried out by using its purified endotoxin for exploring its local or/and systemic effect. In chronic inflammatory diseases of the gut (e.g. in inflammatory bowel diseases), the overproduction of nitric oxide (NO) by the expression of the inducible NO synthase enzyme (iNOS) is cytotoxic, and has a pathological impact [7]. It increases vascular permeability, and leads to vasocongestion [4].

In the present study, we investigated the relation between the expression of iNOS and intestinal inflammation following systemic administration of a purified *H. pylori* endotoxin [5].

2. Materials and methods

2.1. Treatments

We used male Wistar rats (230–250 g). They were fasted overnight, but received water ad libitum. Under transient ether anaesthesia, purified *H. pylori* endotoxin (from A.P. Moran's laboratory, Ireland; 3 mg kg^{-1}) was administered intravenously. In a separate experiment, we administered a bisisothiourea derivative selective iNOS inhibitor (from Wellcome Research Laboratories,

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N-(8-(aminomethyl)-benzyl)acetamidine [1400W], 0.2–1 mg kg^{-1}, s.c.; [2]) concurrently with endotoxin. For the measurement of vascular permeability or iNOS enzyme activity, segments of the jejunum and colon were removed from standard sites 4 h following treatments.

2.2. Intestinal nitric oxide synthase enzyme activity

In this experiment, we used untreated (control) and *H. pylori* endotoxin (3 mg kg^{-1}, i.v.)-treated groups alone or with 1400W (1 mg kg^{-1}, s.c., concurrently with endotoxin) administration. In jejunal and colonic tissues, we measured the calcium-dependent constitutive NO synthase (cNOS) and the calcium-independent iNOS activity by the citrulline assay as described previously [1,2].

2.3. Intestinal vascular permeability

In separate groups of rats, as a measure of inflammation, we examined jejunal and colonic vascular permeability by using the radiolabelled human serum albumin leakage technique as has been established in earlier studies [1]. We used *H. pylori* endotoxin (3 mg kg^{-1}, i.v.)-treated groups alone or with 1400W (0.2–1 mg kg^{-1}, s.c., concurrently with endotoxin) administration.

2.4. Chemicals

We obtained [^{125}I]human serum albumin from IZINTA (Budapest, Hungary) and L-[U-^{14}C]arginine monohydrochloride from Amersham International (UK). All other compounds were from Sigma.

2.5. Statistics

In these studies, the results were expressed as mean±S.E.M. of (n) rats in a group. Data were analysed with the Tukey–Kramer multiple comparisons test, where $P<0.05$ was taken as significant.

3. Results

3.1. Expression of intestinal inducible nitric oxide synthase

Administration of *H. pylori* endotoxin led to the expression of iNOS activity in the jejunum and colon ($n=4$, $P<0.001$) 4 h later, an effect which was abolished by the administration of 1400W ($p=4$, $P<0.001$) in both intestinal tissues (Fig. 1). Basal cNOS activities remained unchanged throughout the experiments ($n=4$, data are not shown).

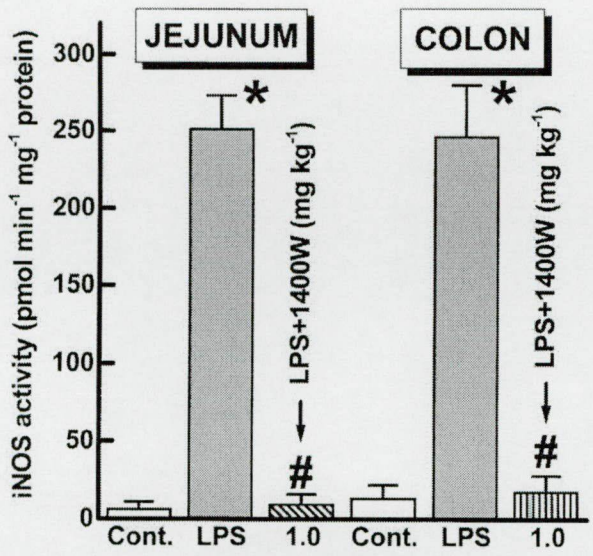

Fig. 1. Expression of iNOS (described in pmol min^{-1} mg^{-1} protein units) in the rat jejunal and colonic tissue 4 h after the administration of purified *Helicobacter pylori* endotoxin (LPS; 3 mg kg^{-1}, i.v.), and its reversal by the selective iNOS inhibitor 1400W (1 mg kg^{-1}, s.c., concurrently with LPS). Data are expressed as mean±S.E.M., where $n=4$ rats in a group. $^{*}P<0.05$ means significant increase in iNOS activity compared with the untreated control (Cont.) group; $^{\#}P<0.05$ means significant decrease in iNOS activity compared to the LPS alone group.

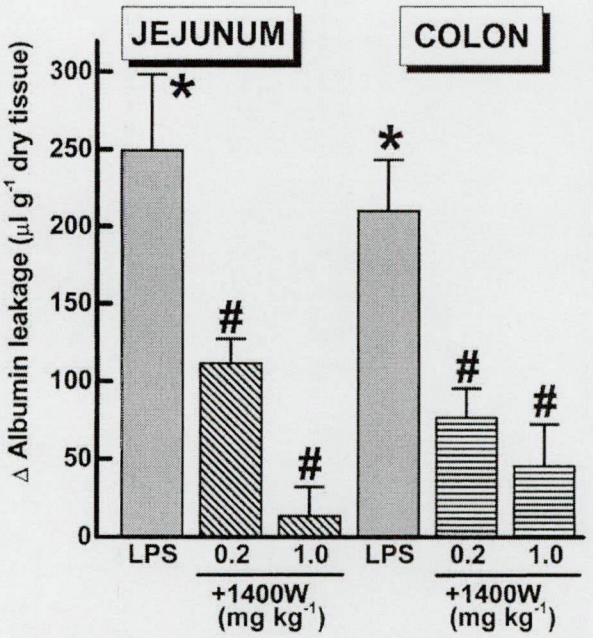

Fig. 2. Provocation of jejunal and colonic albumin leakage (expressed as μl albumin g^{-1} dry tissue) by the administration of a purified *Helicobacter pylori* endotoxin (LPS; 3 mg kg^{-1}, i.v.) 4 h later, and its dose-dependent attenuation by the concurrent administration of a selective iNOS inhibitor (1400W, 0.2–1 mg kg^{-1}, s.c.). Data are expressed as mean±S.E.M., where $n=4$–6 rats in a group. $^{*}P<0.05$ means significant increase in albumin leakage; $^{\#}P<0.05$ means significant decrease in albumin leakage compared to the LPS alone group.

3.2. Intestinal vascular permeability

Administration of *H. pylori* endotoxin (3 mg kg^{-1}, i.v.) alone provoked significant small and large intestinal albumin leakage 4 h later (Fig. 2). Concurrent administration of the selective inhibitor of iNOS, 1400W with *H. pylori* endotoxin dose-dependently attenuated jejunal and colonic albumin leakage after 4 h (83±5% and 94±3% maximal reduction, respectively; $n=4$–6, $P<0.01$) as demonstrated in Fig. 2.

4. Discussion

Our present results support previous observations, when a *H. pylori* extract was administered intravenously, and the expression of iNOS in the duodenum and in its epithelial cells had been found [3]. The expression of iNOS correlated with epithelial cell death, and could be reversed by the administration of the corticosteroid, dexamethasone, and by the treatment with N^G-nitro-L-arginine methyl ester, a non-specific NO synthase blocker at the time of the expression of iNOS [3]. Thus, *H. pylori* extract could damage the intestinal mucosa by its direct toxic effect on epithelial cells, which is mediated by iNOS. The vascular injury correlating with iNOS expression, and their reversal by the selective iNOS inhibitor, 1400W following the challenge with *H. pylori* purified endotoxin in our study suggest that, besides the damage of epithelial cells, microcirculatory dysfunction can also participate in *H. pylori*-related inflammation. Moreover, the induction of NO synthase and microvascular leakage in the jejunum and colon might also reflect *H. pylori* endotoxin-provoked mucosal injury in other parts of the gastrointestinal tract.

In conclusion, *H. pylori* endotoxin is capable of provoking an inflammatory response in the rat intestinal tissue. Systemic release and circulation of *H. pylori* endotoxin could attenuate the mucosal barrier function of the jejunum and colon. This process is mediated by iNOS. Thus, the clinical implication is that selective inhibitors of iNOS might have potential therapeutic benefit in the prevention and treatment of *H. pylori*-associated gastrointestinal mucosal inflammation.

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Endogenous vasopressin increases acute endotoxin shock-provoked gastrointestinal mucosal injury in the rat

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Abstract

Administration of a low dose of endotoxin (from *Escherichia coli*, 3 mg kg^{-1}, i.v.), which does not affect vascular permeability or blood pressure over 1 h, leads to the release of endogenous vasopressin and damage to the mucosal microvasculature. Thus, endogenous vasopressin could be involved in septic shock. In the present study, we investigated the role of endogenous vasopressin in gastrointestinal mucosal injury induced by acute endotoxin shock, which was generated in rats by administering a high dose of *E. coli* endotoxin (50 mg kg^{-1}, i.v.). Tissues were removed 15 min after endotoxin. The vasopressin V_1 receptor antagonist, d[CH_2]$_5$Tyr[Me]arginine–vasopressin (0.2–1 μg kg^{-1}, i.v.), was injected 10 min before endotoxin. Monastral blue (30 mg kg^{-1}, i.v.), which stains damaged vasculature, was injected 10 min before autopsy. Endotoxin reduced systemic arterial blood pressure (from 115 ± 5 to 42 ± 4 mmHg), generated macroscopic and microvascular injury, and elevated plasma vasopressin levels (from 3.4 ± 0.2 to 178 ± 16 pg ml^{-1}). The vasopressin V_1 receptor antagonist reduced this macroscopic injury, and in the vasopressin-deficient Brattleboro rat a similar reduction of gastrointestinal mucosal damage was found. Substantial decreases in endotoxin-induced microvascular damage were observed in each tissue, e.g., the gastric Monastral blue staining was reduced by $47 \pm 3\%$ and $96 \pm 3\%$ ($P < 0.01$) after vasopressin V_1 receptor antagonist treatment and in Brattleboro rats, respectively. Vasopressin, acting through its V_1 receptors, thus appears to be involved in acute endotoxin shock-provoked gastrointestinal injury.

Keywords: Vasopressin; Endotoxin shock; Gastrointestinal mucosa; Microcirculation

1. Introduction

Endotoxin shock is characterised by hypotension, increased vascular permeability and gastrointestinal damage. Bleeding from this gastrointestinal erosions commonly occurs. Once the bleeding becomes manifested, it augments the severe condition and elevates the mortality rate (Robert and Kauffman, 1989; Root and Jacobs, 1991). Although it is important to prevent these stress erosions, it is currently not known how this should be done (Ben-Manachem et al., 1994).

Stress erosions have a multifactorial pathogenesis. Microvascular damage, which leads to hypoxia, has been shown to be an important factor in the generation of gastrointestinal stress erosions (Robert and Kauffman, 1989) as well as in the septic shock (Bone, 1991). In endotoxin shock, the impaired microcirculation may result from a direct injurious action of the lipopolysaccharide component of the bacterial wall on the vascular endothelium (Harlan et al., 1983; Meyrick et al., 1986), or may reflect the formation of tissue-damaging neutrophil- and/or endothelium-dependent vasoactive mediators, such as platelet-activating factor, thromboxanes, leukotrines and/or endothelin (Parillo, 1990; Bone, 1991; Baker et al., 1990; Lefer and Lefer, 1993).

The early release of the potent pituitary nonapeptide, vasopressin, into the plasma has been detected in endotoxaemic states in humans (Dennhardt et al., 1989) and in rats (Baker et al., 1990). Endogenous vasopressin, acting through its pressor (V_1) receptors, has been demonstrated

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to have an aggressive action on the gastrointestinal mucosa during the generation of mucosal stress erosion, e.g., lesions induced by ethanol, indomethacin, reserpine, cold-restraint stress and haemorrhagic shock (László et al., 1991a, 1994, 1996; László and Whittle, 1994). It has also been found that in the early phase of endotoxaemia, i.e., following the administration of a low dose of endotoxin (which does not affect vascular permeability or blood pressure), endogenous vasopressin counteracts the actions of endothelium-derived protective factors, such as constitutive nitric oxide (László and Whittle, 1994). This might suggest that endogenous vasopressin can be involved in septic shock, i.e., in the generation of microvascular dysfunction and damage. The aim of our present study was to investigate the effect of vasopressin on the development of acute gastrointestinal mucosal injury induced by endotoxin shock. For the evaluation of the actions of vasopressin released endogenously, we used a vasopressin-deficient rat strain (homozygous Brattleboro rats; for review see Sokol and Valtin, 1982) and a specific vasopressin V_1 receptor antagonist (for review see László et al., 1991b).

Part of this work has been presented to The British Society of Gastroenterology (László et al., 1993) and to The IVth International Vasopressin Conference (Berlin, Germany, 1993 May).

2. Materials and methods

2.1. Experimental protocol

Female Wistar and homozygous Brattleboro rats (from our breeding farm), weighing 200–220 g, were fasted for 24 h, but received water ad libitum. The animals were anaesthetised with Nembutal (40 mg kg^{-1}, i.p.; Serva, Heidelberg, Germany) and *Escherichia coli* endotoxin (serotype 0111:B4; 50 mg kg^{-1}; Sigma) or saline (control) was injected into the tail vein and the rats were killed 15 min later. This high dose of endotoxin was chosen on the basis of previous studies to generate a mild–moderate grossly visible mucosal injury (Hutcheson et al., 1990). For staining of the injured vasculature, all rats were injected with Monastral blue (30 mg kg^{-1}, i.v.; Sigma) 10 min before autopsy. It should be mentioned here that this colloidal stain penetrates only through damaged microvascular endothelial cells, and not through the basal membrane. The vasopressin V_1 receptor antagonist, d[CH_2]$_5$Tyr[Me]arginine–vasopressin (0.2–1 μg kg^{-1}; Bachem, Germany), was injected i.v. 10 min before endotoxin administration. These doses of the vasopressin V_1 receptor antagonist were selected from previous studies on the basis of their potency to reduce the increase in blood pressure provoked by exogenous vasopressin and to protect the gastric mucosa against damage, i.e., against injury provoked by ethanol, indomethacin, reserpine, cold-restraint stress and haemorrhagic shock (László et al., 1991a, 1994, 1996). Blood pressure in the right carotid artery was monitored with an Elcomatic blood pressure transducer connected to a Grass Polygraph.

2.2. Gross evaluation of lesions

The stomach, duodenum and jejunum were removed, stretched out on cork and photographed. The lesions were examined by an investigator unaware of the nature of the experiment and scored on the basis of a semiquantitative scale (from 0 to III), where 0 = no damage; I = 1–5 petechiae on the mucosal surface; II = more than 5 petechiae and/or mild vasocongested parts; III = severe vasocongestion (Hutcheson et al., 1990).

2.3. Microvascular damage

Two standard-size (3 × 10 mm) pieces of tissue were cut out from standard sites of the stomach, duodenum and jejunum, fixed in formalin and embedded in paraffin. For histological staining of vascular elastic fibres, orcein stain was used. In these sections the background was light brown, blood vessels were dark brown, and injured vessels were blue. The distance of damaged blood vessels (stained with Monastral blue) from the serosal parts was measured with an ocular micrometer, and data are expressed as the maximum average distance of injured (i.e., Monastral blue-stained) blood vessels from the serosal surface in μm.

2.4. Plasma vasopressin level

For the evaluation of changes in plasma arginine–vasopressin levels, in a separate study, rats were decapitated and blood was immediately collected from the wound before (0 min) and after (15 min) endotoxin administration. In control studies plasma arginine–vasopressin levels were measured in intact animals that had been fasted for 24 h, but which had access to water ad libitum. Plasma arginine–vasopressin level was determined with a specific radioimmunoassay system as described previously (László et al., 1994).

2.5. Statistics

For statistical comparisons, analysis of variance with the Bonferroni test was used. Differences were taken as significant when the probability was less than 5%.

3. Results

3.1. Gross evaluation of lesions

Administration of endotoxin (50 mg kg^{-1}, i.v.) reduced blood pressure by 63% (Table 1). As described previously

Table 1
Blood pressure (mmHg) before and during acute (15 min) endotoxin (LPS) shock in rats

Groups—treatments	Before treatment	After treatment
Saline (control)	98 ± 6	95 ± 5
LPS (50 mg kg^{-1})	115 ± 5	42 ± 4[a]
LPS + V_1 antagonist (1 μg kg^{-1})	112 ± 5	50 ± 5[a]
LPS in Brattleboro	105 ± 7	43 ± 6[a]

n = 4–8; mean ± S.E.M.
[a] $P < 0.05$ before treatment groups compared to after treatment groups.

(Hutcheson et al., 1990), this acute endotoxin shock generated gross gastric, duodenal and jejunal mucosal injury in normal (Wistar) rats 15 min later (Fig. 1).

Pretreatment with the vasopressin V_1 receptor antagonist (0.2–1 μg kg^{-1}, i.v.) dose dependently attenuated the mucosal damage of the stomach, duodenum and jejunum provoked by endotoxin in normal (Wistar) rats (Fig. 1). Moreover, endogenous vasopressin deficiency (in homozygous Brattleboro rats) also reduced macroscopic mucosal damage (Fig. 1). The vasopressin V_1 receptor antagonist (1 μg kg^{-1}, i.v.) and vasopressin deficiency did not affect the reduced blood pressure caused by endotoxin shock (Table 1).

3.2. Microvascular damage

Acute endotoxin shock injured intra- and submucosal and subserosal blood vessels in the stomach, duodenum and jejunum within 15 min. It was found that endotoxin administration provoked microcirculatory damage that involved almost the entire wall of the gastrointestinal organs investigated. In endotoxin-treated control rats the subserosal vessels were affected the most. This vascular endothelial injury induced by endotoxin shock was significantly and dose dependently attenuated by pretreatment with the vasopressin V_1 receptor antagonist (0.2–1 μg kg^{-1}, i.v.) and by vasopressin deficiency (in homozygous Brattleboro rats) as demonstrated in Table 2.

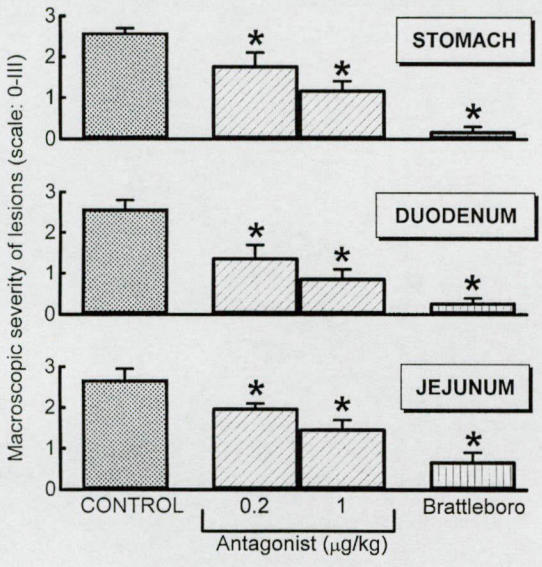

Fig. 1. The protective effect of a vasopressin pressor (V_1) receptor antagonist (Antagonist; d/CH_2/$_5$Tyr/Me/arginine–vasopressin, 0.2–1 μg kg^{-1}, i.v.) and endogenous vasopressin deficiency (in homozygous Brattleboro rats (Brattleboro)) against acute (15 min) endotoxin (LPS, 50 mg kg^{-1}, i.v.) shock-induced gastric, duodenal and jejunal lesions. Macroscopic severity scale: 0–III, determined by an investigator unaware of the nature of the experiment; n = min 4; mean ± S.E.M.; * $P < 0.05$ compared to the LPS-treated (CONTROL) groups.

Table 2
Histological appearance of acute (15 min) vascular damage (assessed by the combination of orcein staining of elastic fibres in blood vessels with the Monastral blue technique for staining damaged vessels) induced by *E. coli* lipopolysaccharide (LPS, 50 mg kg^{-1}), and the protection provided by a vasopressin V_1 receptor antagonist and vasopressin deficiency (homozygous Brattleboro rat)

Groups	Stomach	Duodenum	Jejunum
Saline (control)	0.0	0.0	3.1 ± 0.8[a]
LPS	182.3 ± 7.8	131.5 ± 4.1	56.3 ± 3.2
LPS + V_1 antagonist (0.2 μg kg^{-1})	137.4 ± 4.8[a]	98.0 ± 3.3[a]	37.3 ± 3.8[a]
LPS + V_1 antagonist (1.0 μg kg^{-1})	93.9 ± 5.9[a]	23.9 ± 2.7[a]	13.4 ± 2.2[a]
LPS in Brattleboro	6.1 ± 3.7[a]	1.8 ± 0.9[a]	13.5 ± 4.3[a]

n = min 4; mean ± S.E.M.
[a] $P < 0.05$ compared to LPS-treated groups, data are expressed as the maximum average distance (in μm) of damaged blood vessels (stained with Monastral blue) from the serosal parts of the organs.

3.3. Plasma vasopressin level

Anaesthesia (Nembutal, 40 mg kg^{-1}, i.p.) elevated circulating vasopressin levels. Endotoxin shock caused a further increase in plasma vasopressin. In homozygous Brattleboro rats the plasma vasopressin level was undetectable (Table 3).

4. Discussion

In the present study, the role of endogenous vasopressin in the development of endotoxin shock-provoked acute

Table 3
Changes in plasma arginine vasopressin level during acute (15 min) endotoxin (LPS, 50 mg kg^{-1}, i.v.) shock in rats

Groups	Vasopressin levels (pg ml^{-1})
Control (conscious)	3.4 ± 0.2[a]
Saline (anaesthetised)	43.6 ± 9.0[b]
LPS (anaesthetised)	178.1 ± 16.0[ab]
LPS in Brattleboro (anaesthetised)	> 1.0 ± 0.0

n = 4–8, mean ± S.E.M.
[a] $P < 0.05$ compared to the saline group.
[b] $P < 0.05$ compared to the control group.

gastrointestinal mucosal and microcirculatory damage was evaluated in the stomach, duodenum and jejunum of rats. It was shown that administration of a high dose of endotoxin reduced arterial blood pressure, elevated circulating vasopressin level and generated gastric, duodenal and jejunal macroscopic mucosal and microcirculatory damage in normal (Wistar) rats. Our observations, in relation to changes of plasma vasopressin levels, are in agreement with previous findings in which an increase in circulating vasopressin following anaesthesia or endotoxin administration has been described (Reichlin, 1985; Robertson, 1987; Dennhardt et al., 1989; Baker et al., 1990). Moreover, we found that homozygous Brattleboro rats, which have a congenital deficiency to synthesize vasopressin and consequently have diabetes insipidus (Sokol and Valtin, 1982), were less sensitive to the injurious actions of acute endotoxin shock. Finally, in normal rats, administration of a vasopressin V_1 receptor antagonist dose dependently protected the gastrointestinal microvasculature against the vascular endothelial damage induced by endotoxin. These findings indicate the role of endogenous vasopressin in the generation of acute endotoxin shock-induced gastrointestinal stress erosions.

Microvascular permeability and gastrointestinal damage may respond to initial local changes in blood flow, as vasopressin is a potent vasoconstrictor in the gastrointestinal microcirculation (Burnstock, 1990; Baker et al., 1990; Vanner et al., 1990). Even in endotoxaemic states when the vascular bed is less sensitive to endogenous vasoconstrictor agents, this hormone has been shown to be the most effective vasoconstrictor when compared to other endogenous circulating constrictor agents (Vanner et al., 1990). In addition, vasopressin, via its V_1 receptors, can cause platelet aggregation which may lead the formation of microthrombi in the microcirculation (Filep and Rosenkrantz, 1987). Furthermore, vasopressin may induce gastrointestinal vasoconstriction, vasocongestion or vascular endothelial injury through a thromboxane-dependent mechanism, since it can release thromboxanes from platelets and from vascular tissues (Filep and Rosenkrantz, 1987; Nádasy et al., 1992). The damaging effects of thromboxanes on the gastrointestinal microvasculature following the administration of high doses of endotoxin have been previously reported. In these studies, endotoxin increased the intestinal synthesis of thromboxanes, and the mucosal injury was prevented by thromboxane synthase inhibitors (Boughton-Smith et al., 1989). All of the above described effects of an increased vasopressin formation can lead to an impaired tissue oxygen supply. Indeed, in a recent study vasopressin blockade was shown to improve the impaired oxygen extraction ratio and ketone body availability in the mesenteric circulation following endotoxin administration (Matsuoka and Wisner, 1997).

In conclusion, our current results suggest that endogenous vasopressin has acute injurious actions in the gastrointestinal microcirculation during endotoxin shock. These findings, in conjunction with the beneficial actions of vasopressin deficiency and vasopressin V_1 receptor antagonists in the development of various experimental gastrointestinal lesions (László et al., 1991a, 1994, 1996), indicate the aggressive role endogenous vasopressin has in the generation of gastrointestinal stress erosions in acute septic shock.

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Deleterious action of vasopressin in gastroduodenal ulceration: experimental and clinical observations. Scand J Gastroenterol (Suppl., review) 1998;228:62-67.

Deleterious Action of Vasopressin in Gastroduodenal Ulceration: Experimental and Clinical Observations

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Vasopressin, released from the posterior pituitary and from the vascular endothelium, can cause vasoconstriction and provoke platelet aggregation, leading to an impaired tissue blood supply. In humans with pituitary diabetes insipidus the central release of vasopressin is diminished, and in the Brattleboro homozygous rat there is congenitally no synthesis of this hormone. The gastroduodenal intramucosal vasopressin level is elevated in normal rats following various acute ulcerogenic challenges (after ethanol, reserpine, indomethacin, cold-restraint stress, endotoxin shock and hemorrhagic shock), and vasopressin-deficient rats are less sensitive to these stimuli. In a hospital- and population-based case-control, age-matched retrospective study, the incidence of human gastroduodenal ulceration is significantly higher in the normal population (in whom the release of vasopressin is presumed to be intact) than in the vasopressin-deficient one (central diabetes insipidus patients). In conclusion, endogenous vasopressin plays an aggressive role in development of gastroduodenal ulceration, especially that related to stress.

Key words: Diabetes insipidus; gastric and duodenal mucosa; human vasopressin; peptic ulcer; rat; stress erosion; vasopressin antagonist

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VASOPRESSIN AND GASTROINTESTINAL CIRCULATION

Vasopressin, a potent pituitary nonapeptide, acts on two different types of receptors (1): V_1 (pressor) and V_2 (antidiuretic) receptors. Vasopressin is released from the posterior pituitary in response to numerous stimuli, such as hypovolemia, hyperosmosis and cigarette smoking (2, 3). Vasopressin can also be released locally from the vascular endothelium, in various vascular beds (4–6), including the mesenteric circulation (6). Besides its well-known antidiuretic effect (via its V_2 receptors), vasopressin can cause vasoconstriction (via its V_1 receptors), provoke platelet aggregation (via its V_1 receptors) and release coagulant factors (via its V_2 receptors), leading to an impaired tissue blood supply (2, 3, 7–14).

In the past decade selective peptide and orally effective non-peptide vasopressin V_1 and V_2 receptor antagonists have been developed and used for receptor characterization in animal and in human studies (15–20). For investigations of the role of endogenous vasopressin in the regulation of physiological or pathological processes, the congenitally vasopressin-deficient Brattleboro homozygous rat can be used (21). The pituitary/hypothalamic release of vasopressin is likewise diminished in humans with central (also known as pituitary, cranial or hypothalamic) diabetes insipidus (2, 3, 22). These patients and the Brattleboro homozygous rat exhibit the symptoms of vasopressin deficiency (i.e. polyuria and polydipsia) and are sensitive to the administration of exogenous vasopressin (2, 3, 21, 22).

There is growing evidence that vasopressin plays an important role in the regulation of the gastrointestinal circulation under physiological and pathophysiological circumstances. Physiological doses of vasopressin cause a blood flow reduction in the intestinal tract and vasoconstriction in the superior mesenteric artery (10–13), the latter effect being reversed by a vasopressin V_1 receptor antagonist (13). The existence of vasopressin receptors (23) and the local release of vasopressin from the vascular endothelium (6) in the superior mesenteric artery are also indicative of the physiological importance of this hormone. This artery is extremely sensitive to vasopressin: the minimum amount of the peptide required to decrease the wall diameter is in the order of 1 pg/ml, assessed via direct in vivo and in vitro techniques (24–26). High doses of vasopressin produce vasoconstriction (27, 28), significant ischemia and secondary mucosal damage in the stomach (29). Gastrointestinal mucosal lesions appear following hemorrhage (30–32). On the other hand, in the hemorrhagic state endogenous vasopressin produces mesenteric vasoconstriction (12) and a gastrointestinal blood flow reduction (33), an effect blocked by a vasopressin V_1 receptor antagonist (33). Vasopressin can cause the aggregation of platelets (7–9), and it may provoke gastrointestinal vasoconstriction, vasocongestion or microvascular damage through a thromboxane-dependent mechanism, since it can release

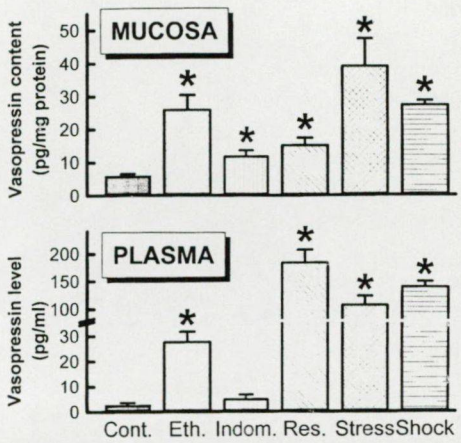

Fig. 1. Gastric intramucosal vasopressin content (upper panel) and plasma vasopressin level (lower panel) following various ulcerogenic challenges, i.e. after ethanol (Eth.; 75%, 1 ml, p.o., 20 min), indomethacin (Indom.; 10 mg/kg, s.c., 4 h), reserpine (Res.; 0.5 mg/kg, s.c., 4 h), cold-restraint stress (Stress; 4 °C, 2-h immobilization) and hemorrhagic shock (Shock; 20-min shock + 20-min reperfusion) exposure in the Wistar rat. *A significant difference at a statistical level of $p < 0.05$ as compared to the controls (Cont.). Data adapted from Reference 44.

thromboxanes from the vascular tissue and the platelets (8, 34), the two mediators interacting synergistically. The thromboxanes exert injurious actions on the gastrointestinal mucosal microcirculation following various challenges; in these models there is an elevated intramucosal synthesis of thromboxanes, and the mucosal damage is prevented by thromboxane synthase inhibitors (35–40). Finally, vasopressin may reduce blood flow by enhancing vascular permeability (41, 42).

The generally accepted main pathogenetic factors in the development of gastroduodenal ulceration are a lowered intramucosal pH, an impaired natural defense mechanism(s), acid hypersecretion, a mucosal energy deficit and gastric hypoxia (32, 43). Since vasopressin exerts action that can correspond with these pathways, we have explored the involvement of endogenous vasopressin in the development of gastroduodenal ulceration.

INCREASED INTRAMUCOSAL AND PLASMA VASOPRESSIN RELEASE FOLLOWING ULCEROGENIC CHALLENGE. INTRAMUCOSAL VASOPRESSIN RECEPTORS

Our aim in these investigations was to examine endogenous vasopressin release following various experimental ulcerogenic challenges. In all of these experiments the investigated species was the Wistar rat.

Gastric and/or duodenal mucosal lesions were induced by means of ethanol (75%, p.o.), indomethacin (10 mg/kg, s.c.), reserpine (0.5 mg/kg, s.c.), cold-restraint stress (2 h at 4 °C), hemorrhagic shock (20 min-shock + 20 min-reperfusion) and endotoxin shock (*E. coli* lipopolysaccharide, 50 mg/kg, i.v.). With the exception of indomethacin induction, the plasma vasopressin level was found to be elevated in all these models (Fig. 1). In contrast, the intramucosal vasopressin content proved to be increased in all the models (Fig. 1). The intramucosal and plasma vasopressin level elevation always occurred simultaneously with, or even preceded, the development of ulceration (44).

It is likely that the increased level of vasopressin in the plasma originates from the posterior pituitary. The fact of the significant elevation of the intramucosal vasopressin content in the indomethacin-induced model, whereas the plasma vasopressin level remained unchanged, might reflect the local, intramucosal generation of vasopressin, since vasopressin-like immunoreactivity has been observed in the rat and human gastric mucosa (45) and vasopressin release from the vascular endothelium has also been demonstrated in various vascular beds (4–6). Our data are in agreement with previous observations that a high dose of orally administered ethanol causes vasopressin release in humans (46) and in rats (47), but in apparent conflict with the established finding that ethanol inhibits the release of vasopressin (2, 3). The paradoxical response of vasopressin release in the ethanol-induced gastric erosion model might possibly reflect the different doses of ethanol administered, which were high (46, 47). It may be mentioned here that the magnitude of the elevation of circulating vasopressin following the intravenous administration of graded doses of vasopressin in combination with oral ethanol has been shown to correlate with the increasing

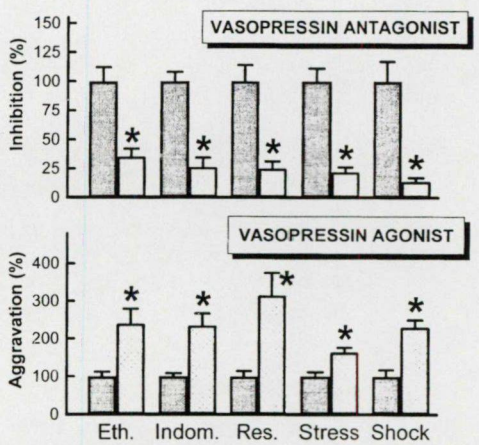

Fig. 2. Percentage inhibition by a vasopressin antagonist ($d(CH_2)_5Tyr(Me)AVP$, hatched columns in upper panel) and potentiation by a vasopressin agonist (lysine-vasopressin, hatched columns in lower panel) on pretreatment (30 min before challenges) of gastric mucosal lesions induced by ethanol (Eth.; 75%, 1 ml, p.o., 1 h), indomethacin (Indom.; 10 mg/kg, s.c., 4 h), reserpine (Res.; 0.5 mg/kg, s.c., 8 h), cold-restraint stress (Stress; 4 °C, 2-h immobilization) and hemorrhagic shock (Shock; 20-min shock + 20-min reperfusion) in the Wistar rat. *A significant difference at a statistical level of $p < 0.05$ between the control (Cont.; gray columns) and treated groups. Data adapted from References 27, 44 and 51.

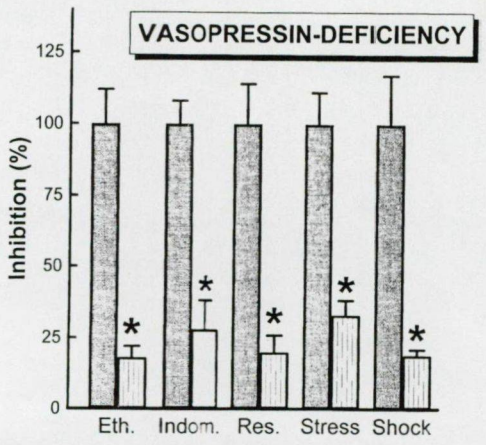

Fig. 3. Protection of the gastric mucosa (expressed as percentage inhibition of lesions) by a vasopressin deficiency (in the Brattleboro homozygous rat; shaded columns) against damage induced by ethanol (Eth.; 75%, 1 ml, p.o., 1 h), indomethacin (Indom.; 10 mg/kg, s.c., 4 h), reserpine (Res.; 0.5 mg/kg, s.c., 8 h), cold-restraint stress (Stress; 4 °C, 2-h immobilization) and hemorrhagic shock (Shock; 20-min shock + 20-min reperfusion). *A significant inhibition at a statistical level of $p < 0.05$ in comparison with the controls (gray columns). Data adapted from References 27 and 44.

severity of the ethanol-induced gastric hemorrhagic lesions (47). Body compression and restraint stress also increase the vasopressin level in the plasma (48, 49), and the gastric mucosal vasopressin content is elevated in the activity-stress-induced ulcer model (50).

The above findings led us to suspect that, besides the superior mesenteric artery (23), the gastric mucosa also contains vasopressin receptors. Indeed, by using a radioiodinated vasopressin V_1 receptor antagonist, we detected high-affinity vasopressin binding sites by receptor autoradiography in the intramucosal vasculature in the rat stomach (44).

EFFECTS OF VASOPRESSIN AGONISTS AND ANTAGONISTS IN ULCER MODELS

In this series of studies, we explored whether inhibition of the action of endogenous vasopressin (by vasopressin antagonists) or the administration of exogenous vasopressin modifies the development of experimental gastrointestinal mucosal damage.

Administration of a vasopressin V_1 receptor antagonist significantly reduced the macroscopic and histopathological gastric mucosal damage induced by ethanol, indomethacin, reserpine, cold-restraint stress, hemorrhagic shock and endotoxin shock (27, 44, 51–53), as demonstrated in Fig. 2. The V_1 antagonist was found to have no effect on the intragastric pH (27), which suggests that the antiulcerogenic action of V_1 antagonists does not relate to gastric acid secretion. It was also demonstrated that the V_1 antagonist protected the duodenal mucosa in the hemorrhagic shock and endotoxin shock-induced models (52, 53). Finally, V_1 receptor antagonists exerted significant beneficial actions on jejunal, ileal and colonic microvascular injuries following the administration of high and low doses of endotoxin (53, 54).

In contrast to the beneficial effect of vasopressin antagonists, the exogenous administration of a vasopressin agonist, lysine-vasopressin (1 μg/kg, s.c.), aggravated the gastroduodenal mucosal injury provoked by ethanol, indomethacin, reserpine, cold-restraint stress, hemorrhagic shock and endotoxin shock (27, 44, 52), as shown in Fig. 2. Injection of this ulcerogenic dose of lysine-vasopressin into rats caused a significant reduction in gastric blood flow (27) and simultaneously provoked an increase in intragastric pH (27). This blood flow reduction in the stomach might originate from a vasopressin-mediated vasoconstriction and/or vasocongestion (2, 3, 7–14) and may explain the decreased gastric acid secretion observed following vasopressin administration (27, 55, 56).

VASOPRESSIN-DEFICIENT RATS ARE LESS SENSITIVE TO ULCEROGENIC STIMULI

Apart from the use of vasopressin antagonists, the role of endogenous vasopressin in various physiological or pathological processes can be investigated in the congenitally vasopressin-deficient Brattleboro homozygous rat (21). Following the oral administration of ethanol, we found that the extent of macroscopic and histologic mucosal injury was significantly less in the stomach of the Brattleboro homo-

Table I. Data on the incidence of gastroduodenal ulceration among the normal-vasopressin population and among vasopressin-deficient subjects with central diabetes insipidus in the 20–70 years age group in Hungary

Distribution of gastroduodenal ulceration (GDU)[a]	No. of normal-vasopressin subjects[b]	No. of diabetes insipidus patients
No. of GDU-negative subjects	6 532 007	811
No. of GDU-positive cases	148 198	4
Percentage incidence	2.22	0.49*

[a] Acute or chronic gastric and/or duodenal ulceration, with or without a complication (bleeding and/or perforation), currently existing or featuring in the case history.

[b] The total population in Hungary without diabetes insipidus in the 20–70 years age group (numbers on 1 January 1995). Significantly different at a statistical level of $*p < 0.005$.

zygous rat than in the normal Wistar rat (Fig. 3). The vasopressin deficiency in the former strain protected the deeper layer of the gastric mucosa and inhibited mucosal microvascular injury (assessed by the Monastral blue technique) following ethanol challenge. Administration of exogenous vasopressin to the Brattleboro rat enhances the gastric mucosal injury to the level found in a normal strain with intact vasopressin secretion. This dose of vasopressin also provoked a significant reduction in gastric blood flow. The protection against ethanol damage in the Brattleboro rat was not related to an unusual gastric emptying or gastric acid secretion in this special strain, since there were no differences in these parameters between normal and vasopressin-deficient rats (27).

Further studies revealed that the gastric mucosa of the Brattleboro homozygous rat is less sensitive to other ulcerogenic stimuli: the gross and microscopic gastric damage was significantly attenuated following reserpine or indomethacin administration, and during cold-restraint stress, hemorrhagic shock and endotoxin shock (44, 51), as demonstrated in Fig. 3. Similarly, as observed in the ethanol-induced model, the congenital vasopressin deficiency protected the deeper layer of the mucosa after these stimuli (44, 51). Moreover, the vasopressin deficiency had a beneficial effect during the generation of macroscopic and histologic mucosal damage and microvascular injury induced by hemorrhagic shock and endotoxin shock in the duodenum and jejunum (52, 53).

LOWER INCIDENCE OF GASTRODUODENAL ULCERATION AMONG VASOPRESSIN-DEFICIENT HUMANS

The observations that various ulcerogenic challenges increased the intramucosal vasopressin level in normal rats (44), whereas the gastroduodenal mucosa of the congenitally vasopressin-deficient Brattleboro homozygous rat was less sensitive to these stimuli (27, 44, 52, 53), led us to seek a link between our experimental findings and human ulcerogenesis. Accordingly, we evaluated the incidence of gastroduodenal ulceration among central diabetes insipidus patients (persons with an impaired vasopressin release) in comparison with that in the normal population (in whom the release of endogenous vasopressin is presumed to be intact) by performing a hospital- and population-based case-control, age-matched retrospective study (57). Data on all patients (with gastroduodenal ulceration and/or with a vasopressin deficiency) aged 20–70 years who were hospitalized in Hungary between 1 January 1992 and 1 October 1995 were compared with the data on the normal Hungarian population in the same age group (number on 1 January 1995).

The incidence of gastroduodenal ulceration was significantly higher in the normal population than in the vasopressin-deficient one (odds ratio: 4.60 with 95% confidence interval: 1.9–11.2; $p = 0.001$), as demonstrated in Table I. An intact endogenous vasopressin release therefore appears to favour the development of ulceration of the human gastroduodenal mucosa relative to that in a vasopressin-deficient state, as in the Brattleboro rat (27, 44, 52, 53).

CONCLUSIONS

The release of vasopressin into the circulation has been demonstrated in animal models of gastroduodenal ulceration, and there are also strong grounds for suspecting the local intramucosal secretion of the hormone. The existence of intramucosal vascular vasopressin receptors in the stomach lends further support to the physiological and pathophysiological actions of the peptide in regulation of the gastrointestinal circulation. This vasopressin release mediates pathological processes in the generation of gastrointestinal mucosal injury, since vasopressin antagonists and a vasopressin deficiency provide a protective effect in various experimental models of gastrointestinal ulceration. Although endogenous vasopressin seems to have an important role in the generation of the gastrointestinal mucosal injury induced by various ulcerogenic challenges, this hormone is not the only factor involved: the macroscopic and histologic severity of the lesions was not related to the plasma or intramucosal vasopressin levels in the ethanol and cold-restraint stress models (44). It is strongly suspected that endogenous vasopressin interacts with other damage-provoking mediators (thromboxanes, leukotrienes, platelet-activating factor, etc.) in the pathogenesis of ulceration of the gastrointestinal mucosa (40, 54, 58).

The lower incidence of gastrointestinal ulceration among vasopressin-deficient patients suggests the pathological importance of endogenous vasopressin release in the development of human mucosal damage. Gastrointestinal mucosal stress erosions commonly appear among critically ill patients in intensive care units after a number of acute conditions, such as severe trauma, burns, septic shock, hemorrhagic shock and cardiogenic shock or injury to the central nervous system. Once hemorrhage becomes manifest, the severity of the condition is reflected in the mortality rate, which approaches 50% (32). Although it would be of high significance, the problem of how to prevent the development of such erosions is unsolved (59). In these hypovolemic states the release of endogenous vasopressin is highly probable (2, 3, 22). The experimental and clinical observations lead us to conclude that vasopressin antagonists might be of therapeutic importance in the prevention of the generation of gastrointestinal stress erosions.

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Vasopressin deficiency decreases the frequency of gastroduodenal ulceration in humans

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Abstract — Vasopressin is a stress hormone released from the posterior pituitary. In humans suffering from central diabetes insipidus, this release of vasopressin is diminished. It was shown previously that the congenutally vasopressin-deficient Brattleboro homozygous rat is less sensitive to various ulcerogenic stimuli. In this study, we investigated the incidence of gastroduodenal ulceration in vasopressin deficient patients. Data on patients aged 20–70, hospitalized in Hungary between 1992 and 1995 were compared with those on the total population in this age group (6 681 020 in 1994). Subjects with central diabetes insipidus were selected separately (815 cases). Gastroduodenal ulceration was compared in subjects with an intact vasopressin release and vasopressin-deficient patients. The frequencies of gastroduodenal ulceration were also examined separately in male and female subjects. In the total population, the frequency of gastroduodenal ulceration was lower in vasopressin-deficient cases (2.22% versus 0.61%; $P < 0.005$). Among normal-vasopressin subjects, males have a higher risk of gastroduodenal ulceration than females (3.04% versus 1.46%, respectively; $P < 0.001$). Among vasopressin-deficient subjects, a similar male:female ratio was observed, but it was not significant ($P = 0.36$). In comparison to the normal-vasopressin population, the incidence of gastroduodenal ulceration was reduced among vasopressin-deficient males and females by 77% ($P < 0.01$) and by 82% ($P < 0.05$), respectively. In conclusion, endogenous vasopressin has a significant harmful action towards the human gastroduodenal mucosa. Peptide and non-peptide vasopressin receptor antagonists might have a potential therapeutic benefit in the treatment (as an adjuvant) and prevention of gastroduodenal ulceration. © 2000 Elsevier Science Ltd. Published by Éditions scientifiques et médicales Elsevier SAS

gastric ulcer / duodenal ulcer / ulcer disease / vasopressin / vasopressin receptor antagonist / diabetes insipidus

1. Introduction

Gastroduodenal ulceration is a common disease. It is known that aggressive (e.g., gastric acid, pepsin) and protective (e.g., mucosal blood flow, prostaglandins) factors act in the development of acute or chronic mucosal lesions, and an imbalance between them plays a key role in the occurrence of the disease [15].

In animals, endogenous vasopressin has been demonstrated to act as an aggressive factor towards the gastroduodenal mucosa, since the vasopressin-deficient Brattleboro homozygous rat [16] is less sensitive to various ulcerogenic stimuli than the rat with normal-vasopressin secretion [5, 6, 8].

In humans, suffering from central diabetes insipidus, the secretion/release of vasopressin from the hypothalamus/posterior pituitary is impaired. These patients are ideal subjects of the investigation of the role of endogenous vasopressin in generation of various diseases, in which this stress hormone might possibly have a pathogenetic action. Accordingly the aim of the present study was to evaluate the incidence of gastroduodenal ulceration among vasopressin-deficient human subjects in comparison to the normal-vasopressin population.

* Correspondence and reprints

2. Materials and methods

2.1. Study groups

Two groups of human subjects aged between 20 and 70 years were selected: the vasopressin-deficient and the non-deficient groups. The vasopressin-deficient cases suffered from central diabetes insipidus, while the non-deficients were taken as the normal population in Hungary in 1994 (data on 01.01.1995). All central diabetes insipidus cases hospitalized in Hungary between 01.01.1992 and 01.10.1995 were selected. Additionally, the subjects in the two groups were examined on the basis of their sex.

2.2. Cases with gastroduodenal ulceration

All patients aged 20–70 years hospitalized in Hungary between 01.01.1992 and 01.10.1995 with gastroduodenal ulceration (including acute or chronic gastric and/or duodenal ulceration with or without

Table I. Data on normal-vasopressin and vasopressin-deficient humans aged 20–70 years in Hungary.

	Male		*Female*		*Male + female*	
	Normal	DI	Normal	DI	Normal	DI
Number	3 213 613	424	3 467 407	391	6 681 020	815
Hosp.	1 972 000	562	2 977 599	507	4 949 599	1069

Normal, subjects with intact vasopressin release.
DI, subjects with diabetes insipidus (vasopressin-deficient).
Number, number of persons in this age group in Hungary in 1994 (data on 01.01.1995).
Hosp., total number of hospitalizations of subjects in this age group in Hungary between 01.01.1992 and 01.10.1995.

complication (bleeding and/or perforation) currently or in the case history) were selected. In each group, patients were taken as vasopressin-deficient if the appearance of diabetes insipidus preceded the onset of gastroduodenal ulceration.

2.3. Comparisons

The frequencies of gastroduodenal ulceration were compared in the vasopressin-deficient (central diabetes insipidus) patients and the non-deficient population. The male and female populations were also compared.

2.4. Statistics

For statistical comparisons the Mantel-Haenszel Chi-square test and for relative risks the odds ratio (OR) with 95% confidence interval (CI) were used, where appropriate. $P < 0.05\%$ was taken as indicating statistical significance.

3. Results

3.1. Study groups

The male:female ratio is demonstrated in *table I.* The male:female ratio in the vasopressin-deficient and normal-vasopressin population was approximately 50%, being 48 versus 52%, respectively among vasopressin-deficients and 52 versus 48%, respectively among cases with intact vasopressin release.

3.2. Cases with gastroduodenal ulceration

The risk of gastroduodenal ulceration was significantly higher in the normal-vasopressin population as compared to the vasopressin-deficient population (OR: 4.60 with 95% CI: 1.88–11.24; $P < 0.005$), as demonstrated in *figure 1.* Among normal-vasopressin subjects, males have a higher risk of gastroduodenal ulceration than females (OR: 2.12 with 95% CI: 2.10–2.15; $P < 0.001$). A higher male:female ratio was observed in vasopressin-deficient cases, but it was not significant (OR: 2.78 with 95% CI: 0.32–24.44; $P = 0.36$). In comparison to the normal-vasopressin population, the incidence of gastroduodenal ulceration was reduced among vasopressin-deficient males and females by 77% ($P < 0.01$) and by 82% ($P < 0.05$), respectively, as shown in *figure 1.* The risk to acquire gastroduodenal ulceration was 4.40 (OR with 95% CI: 1.56–12.43) and 5.76 (OR with 95% CI: 1.02–32.59) among normal-vasopressin males and females, respectively, compared to vasopressin-deficient ones.

4. Discussion

In this work, the incidence of acute and chronic gastric and/or duodenal ulceration has been found to be lower in vasopressin-deficient humans than in the normal-vasopressin population aged 20–70 years. This result might indicate the pathogenic role of endogenous vasopressin in development of gastroduodenal ulceration.

In various rat models of gastroduodenal ulceration (i.e., in the ethanol-, reserpine-, stress- and indomethacin-induced gastric, and in the hemorrhagic shock-provoked gastroduodenal one), the intramucosal and plasma vasopressin levels were detected to be elevated [3, 7, 8]. It should be noted here, that the measurement of intramucosal and plasma vasopressin looks like indicated in humans with and without acute or chronic gastroduodenal ulceration. Endogenous vasopressin may act on its intramucosal pressor (V_1) receptors, since previously on gastric microvessels of the rat such kind of receptors have been demonstrated [8]. The existence of vasopressin receptors in the superior mesenteric artery [17], in conjunction with the finding that this blood vessel is highly sensitive for vasopressin both in vitro and in vivo [1, 2, 4, 13], suggests that this hormone can also act in the macrocirculation of the gut. Finally, it is known that administration of exogenous vasopressin derivatives is useful in the treatment of gastrointestinal bleeding of humans [15].

Endogenous vasopressin, released from the posterior pituitary and from the vascular endothelium [10-

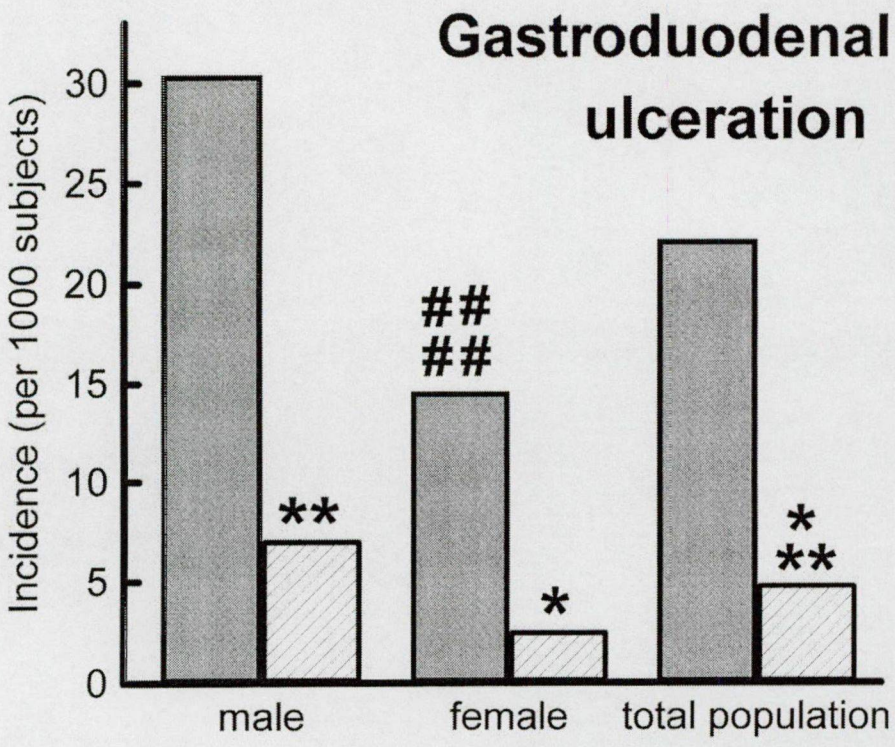

Figure 1. Incidence (per 1 000 subjects) of gastroduodenal ulceration (including acute and chronic gastric and/or duodenal ulceration with or without complication, such as bleeding and/or perforation) among the normal Hungarian population aged 20–70 years (males, females and total population) with an intact vasopressin release (gray column) or with a vasopressin deficiency (hatched column). Statistical significance: * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.005$ for normal-vasopressin versus vasopressin-deficient cases, and ##### $P < 0.001$ for males versus females.

12], may generate vasoconstriction and platelet aggregation (via its V_1 receptors), and release coagulant factors (via its antidiuretic V_2 receptors), leading to a reduced blood flow and a consequent hypoxia in the gastroduodenal mucosa. The reduction of mucosal oxigenation might attenuate the protective mechanisms of the gut and sensitize it to the damaging effect of other well-known aggressive factors, such as gastric acid and pepsin [15]. Indeed, administration of vasopressin V_1 receptor antagonists elevates blood flow in the superior mesenteric artery [14] and protects against various ulcerogenic challenge [5, 6, 8, 9]. Although in a previous study, a selective V_2 receptor antagonist has been reported to be ineffective against ethanol-induced gastric hemorrhagic erosions in the rat [6], but the involvement of this type of vasopressin receptor seems not to be excluded in generation of gastroduodenal ulceration, since a non-selective vasopressin receptor antagonist (V_1/V_2 antagonist) was shown to be more potent in the ethanol model than the selective V_1 receptor antagonist alone (our unpublished result).

In conclusion, endogenous vasopressin seems to have a significant harmful action towards the human mucosa in generation of gastroduodenal ulceration. Peptide and orally effective non-peptide vasopressin receptor antagonists may have a potential therapeutic benefit in the prevention and treatment (as an adjuvant) of gastroduodenal ulceration.

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Inducible nitric oxide synthase attenuates chronic colitis in HLA-B27/human β2-microglobulin transgenic rats

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ABSTRACT. Rats transgenic for HLA-B27/human β_2-microglobulin develop a spontaneous multisystem inflammatory disorder that closely mimicks human spondyloarthropathies. Prominent features of this disorder are gut inflammation that predominates in the colon, and arthritis. Several mediators such as IFN-γ, IL-1β, TNF-α, and inducible nitric oxide synthase (iNOS) have been found increased in the inflamed colonic mucosa. In the colon of HLA-B27 transgenic rats, iNOS is predominantly expressed by epithelial cells, and iNOS transcripts are detected in the hip cartilage of those rats, but not in nontransgenic littermates. The role of iNOS in this disorder was evaluated by administering the corticosteroid dexamethasone, or the NOS inhibitor L-N^6-(1-iminoethyl)lysine (L-NIL) to HLA-B27 transgenic rats with established disease. Treatment with dexamethasone attenuated some aspects of gut inflammation, although it had no effect on iNOS expression. In contrast, treatment with L-NIL effectively inhibited iNOS activity, and resulted in an increase in colitis. Cytokine transcripts in the colon were modified by these treatments: IFN-γ and IL-1β were decreased after dexamethasone treatment, whereas administration of L-NIL resulted in decreased IFN-γ, and TNF-α. A trend towards increased IL-1β expression was observed which could have contributed to the L-NIL pro-inflammatory effect. These results suggest that iNOS exerts a protective effect on colitis, in the inflammatory disorder of HLA-B27 transgenic rats.

Keywords: rodent, inflammation, nitric oxide, cytokines, *in vivo* animal models.

INTRODUCTION

The HLA-B27 class 1 major histocompatibility complex allele is strongly associated with the spondyloarthropathies, a group of human disorders characterized by the combination of osteoarticular, skin and gut inflammatory manifestations [1]. Several lines of rats transgenic for HLA-B27 and human β_2-microglobulin develop a spontaneous multisystem inflammatory disease that strikingly resembles human spondyloarthropathies, by combining arthritis with colitis and psoriatic skin lesions [2]. The immune system is critically involved in the pathogenesis of this disease in HLA-B27 transgenic rats, which is transmittable to nontransgenic rats by bone marrow graft, and requires the presence of mature T cells for its development [3, 4]. Based on these findings, it is hypothesized that disease in HLA-B27 transgenic rats arises as a consequence of interaction between an antigen-presenting cell expressing high levels of HLA-B27, and peripheral T lymphocytes [3, 4]. The presence of a normal gut flora is also required for the gut and the joint disease to develop [2].

Mediators implicated in the pathogenesis in the HLA-B27 transgenic rat model have been most extensively studied in the gut mucosa. The profile of cytokines expressed in the inflammed colonic mucosa is characterized by a prominent early increase in IFN-γ and IL-2, suggesting a Th1-mediated predominant response [2]. In established disease, pro-inflammatory mediators such as IL-1α, IL-1β, TNF-α, macrophage inflammatory protein 2, and myeloperoxidase (MPO) are also expressed in the inflammed colonic mucosa [5, 6]. Elevated plasma levels of nitrite/nitrate are consistent with an increased production of nitric oxide (NO) in this model [7] and the inducible nitric oxide synthase isoform (iNOS) was detected in the colonic mucosa of affected B27 transgenic rats by reverse transcriptase-polymerase chain reaction (RT-PCR) and by enzymatic activity assay [5, 6].

We previously reported that administration of murine recombinant IL-10 to HLA-B27 transgenic rats with established disease had no influence on the clinical or histopathological course of the disease. However,

IL-10 efficiently inhibited production of IFN-γ, TNF-α, and IL-1β in the gut mucosa, and conversely increased colonic iNOS expression (5; M. Breban and D. Lamarque, unpublished results). This result suggested that iNOS could play a pathogenic role in this model, by the production of NO-derived injurious products such as peroxynitrites, or by altering the balance between pro- and anti-inflammatory cytokine production [8, 9]. In the present study, we tested this hypothesis by administering to HLA-B27 transgenic rats with established disease, dexamethasone, which can prevent the expression of iNOS [10, 11], and the iNOS inhibitor, L-N^6-(1-iminoethyl)lysine (L-NIL) [12], considered to offer selectivity for the iNOS isoform.

MATERIALS AND METHODS

Rats. The HLA-B27 transgenic rat line 33-3, on a Fisher (F344) background [2], was maintained by breeding 33-3 females with nontransgenic F344 males, and typing offsprings for the B27/hβ_2m transgene by PCR amplification of tail genomic DNA with an HLA-B27 specific pair of primers RT11 and RT12 [13]. All rats were maintained under conventional conditions at Cochin University Hospital animal facility.

Treatment. Dexamethasone was administered s.c. daily (study 1). L-NIL (Alexis, Paris, France) was administered s.c. twice daily (study 2). Sex- and age-matched rats of the 33-3 line with overt disease (6 rats per group, 11-39 weeks old: study 1; 8 rats per group, 17-21 weeks old: study 2), and control F344 littermates (4 rats per group in each study) received either active compound or vehicle.

Clinical evaluation. Rats were scored three times each week for diarrhea and arthritis, on a scale ranging from 0 (normal) to 4 for each of these symptoms, by the same examiner unaware of the treatment. The average daily total of these scores was calculated each week.

Macroscopic and histological evaluation of organs. After completion of treatment, rats were sacrificed. Mesenteric lymph nodes (MLN) were weighed and colon weight/length was measured. Tissue samples from the caecum, proximal colon and distal colon, were fixed in formalin, embedded in paraffin, sectioned, stained with hematoxylin, eosin, safran, and coded for blind examination. Sections were scored for ulceration density (absent: 0; covering < 1/3 of examined area: 1; covering 1/3 to 2/3 of examined area: 2; covering > 2/3 of examined area: 3), ulceration depth (limited to mucosa: 1; reaching submucosa: 2; reaching muscularis propria: 3) and extent of polymorphonuclear and mononuclear cells infiltration in the chorion (absent: 0; limited to mucosa: 1; reaching submucosa: 2; reaching muscularis propria: 3). The global histological score was the sum of the values obtained for the caecum, the proximal colon, and the distal colon per rat.

Immunohistochemistry. An antigen retrieval method was performed on 5 μm thick, deparaffinized sections of colon, using micro-wave oven heating (3 x 5 min) in 6 mM citrate buffer pH 6. Sections were then incubated for 10 min in 3% hydrogen peroxide to block endogenous peroxidase activity, followed by 1 hour with anti-iNOs monoclonal antibody, dilution 1/40 (Transduction Laboratories, Lexington, UK). A three-step indirect process based on avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) and 3,3-diaminobenzidine (Sigma, St-Quentin Fallavier, France) as chromogen, was applied to reveal as a brown precipitate the positive reaction. Negative controls were obtained by omitting the primary antibody.

Western immunoblot analysis. The whole colonic mucosa or colonic epithelial cells, isolated by the standard method [14], were homogenized in buffer (2 mM Tris 7-9, 50 mM mannitol, 100 μM phenylmethylsulfonyl, 2 μM leupeptin, 0.5 mu/ml aprotinin, 0.5% triton X100). Spleen homogenate from rat challenged i.v. with *Escherichia coli* lipopolysaccharide (LPS serotype O111:B4; Sigma) was used as a positive control for iNOS. Homogenates were sonicated twice for 10 sec on ice and spun (21,000 *g* at 4° C, 15 min). Aliquots of 20 μg of total cellular protein were denatured by boiling v/v with 20 mM Tris 7-9, 2 mM EDTA, 2% SDS, 10% β-mercaptoethanol, 20% glycerol. Samples were electrophoresed on 7.5% SDS-polyacrylamide gel, and transferred to nitrocellulose membrane (Amersham, Little Chalfont, UK). After blocking with PBS (pH 7.4), 0.25% tween 20 (v/v) and 5% non-fat dried milk, the membrane was probed with anti-iNOS polyclonal antibody (sc-651; Santa Cruz, Santa Cruz, CA, USA) for 1 hour, washed with PBS-tween 20 and then incubated with horseradish peroxidase-conjugated second antibody for 1 hour at room temperature. Membranes were developed, using an enhanced chemiluminescence system (Amersham) and exposed to Hyperfilm (Amersham). Films were scanned on a GS-700 Imaging Densitometer (Bio-Rad, Hercules, USA) and analysed using the Molecular Analyst Software (Bio-Rad).

MPO assay. MPO activity was determined as described, with minor modifications [15]. Segments of colon were homogenized in ice-cold phosphate buffer containing 0.5% hexadecyltrimethylammonium-bromide, freeze-thawed three times and centrifuged (10,000 *g*, 15 min, 4° C). Supernatant was mixed with phosphate buffer containing 0.167 mg/ml of O-adenosine dihydrochloride and 5×10^{-4}% hydrogen peroxide and assayed spectrophotometrically (500 nm). MPO activity was expressed in mU/g of tissue.

Determination of iNOS enzymatic activity. Activity of iNOS was estimated by measuring the conversion of L-[^{14}C]-arginine monohydrochloride to L-[^{14}C]-citrulline, based on a previously described method [16]. Briefly, a colon sample was homogenized in HEPES buffer containing protease inhibitors. After centrifugation (10,000 *g*, 30 min, 4° C), an aliquot of supernatant was added to prewarmed buffer containing (final concentration) potassium phosphate (50 mM;

pH = 7.4), valine (50 mM), $MgCl_2$ (1 mM), $CaCl_2$ (200 µM), DL-DTT (1 mM), L-citrulline (1 mM), NADPH (0.3 mM), FAD (3 µM), FMN (3 µM), BH4 (3 µM), L-[^{14}C]-arginine monohydrochloride (15.5 nM; Amersham, Les Ulis, France) and incubated for 10 min at 37° C. The incubation was terminated by the binding of arginine, by addition of a 1:1 suspension of Dowex (AG 50W-8; Sigma) in water. Supernatant was taken for scintillation counting. Activity of iNOS was that which was inhibited by *in vitro* incubation with the NO synthase inhibitor N^G-monomethyl-L-arginine (300 µM; Sigma) but not with EGTA (1 mM), and was expressed as pmol.min^{-1}.mg^{-1} protein [17].

RT-PCR. Total RNA was isolated with the TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and reverse transcribed into complementary DNA (cDNA) using AMV reverse transcriptase. Amplification was carried out in a 100 µl volume containing 100 ng cDNA, 125 µM of each 2'-deoxynucleoside 5'-triphosphate, 2 U of Taq DNA polymerase, 50 ng of each primer and 10 µl of 10 X PCR buffer. Primer sequences and the annealing temperature used for β-actin, CD3, IL-1β, TNF-α, and IFN-γ were as described [5]; other primers were: IL-10 sense 5'-TGC CTT CAG TCA AGT GAA GAC T-3', antisense 5'-AAA CTC ATT CAT GGC CTT GTA-3', Tm = 55° C; iNOS sense 5'-CAA TCC ACA ACT CGC TCC AA-3', antisense 5'-GCT ACA CTT CCA ACT CAA CA-3', Tm = 57° C. Amplification conditions consisted of 3 min at 92° C followed by 35 cycles of denaturation at 94° C for 30 sec, annealing for 30 sec, extension at 72° C for 40 sec, and by final elongation at 72° C for 10 min. Fifty microliters of PCR product were electrophoresed on agarose gel. Negative pictures of the gels were taken for quantitation with a Personal Densitometer and the Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA, USA). Data are expressed in arbitrary units, as the ratio between optical density for the specific cDNA and the one for β-actin on the same sample.

Statistics. The data are expressed as mean ± SEM, unless otherwise stated. The statistical significance of differences was tested with the Mann Withney nonparametric U test. *P* values less than 0.05 were considered significant.

RESULTS

Localization of iNOS expression in HLA-B27 transgenic rats. Expression of iNOS was reproducibly detected by Western Blot analysis (Figure 1), and by immunohistochemistry (Figure 2) in the whole colon of HLA-B27 transgenic rats with established colitis, but not in the colon of nontransgenic F344 littermates. Immunohistochemistry performed on the colon of 33-3 rats, demonstrated the predominance of iNOS expression in the epithelium over the chorion (Figure 2). This result was consistent with the strong detection of iNOS in isolated colonic epithelial cells from 33-3 rats (Figure 1). In addition, RT-PCR analysis of several tissues was performed in HLA-B27 transgenic rats in parallel with nontransgenic littermates. These experiments demonstrated the presence of iNOS mRNA in the hip cartilage of 7 out of 10 HLA-B27 transgenic

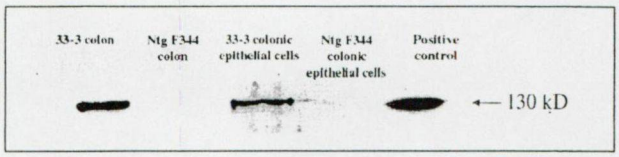

Figure 1
Western-blot analysis of iNOS in the whole colon and in isolated colonic epithelial cells from the HLA-B27 transgenic rat of the 33-3 line with established disease, and from a non-transgenic (Ntg) F344 littermate.
Spleen homogenate from rat challenged i.v. with LPS was used as a positive control of iNOS expression. This result is representative of 4 separate experiments.

Figure 2
Detection of iNOS by immunohistochemistry performed on the colonic mucosa.
A: colon from an HLA-B27 transgenic rat of the 33-3 line with established disease. The section shows chronic colitis with ulceration, cryptic abcess and marked infiltration of inflammatory cells, and immunohistochemical localisation of iNOS as diffuse, dark staining, localized mainly in the apical epithelium (original magnification x 40). Positive detection of iNOS was observed in colon from 8 different 33-3 rats examined. **B:** normal colon from non-transgenic F344 littermate. The section shows no epithelial cells or lamina propria staining (original magnification x 100). A positive staining of ganglionic cells from Meissner's plexus can be noted (arrows).

rats studied, in addition to the colon (data not shown), but not in this tissue from nontransgenic littermates, nor in any the following tissues from affected 33-3 or from nontransgenic F344 rats that were examined: stomach, ileum, heart, eye, synovial tissue from arthritic and nonarthritic knees (specimens from 2 to 5 rats for each tissue were examined; data not shown).

Dexamethasone improves spontaneous disease in HLA-B27 transgenis rat but fails to inhibit iNOS expression. In study 1, groups of 33-3 rats and nontransgenic littermates were treated for 4 weeks with either dexamethasone (0.25 $mg.kg^{-1}.day^{-1}$), or vehicle. None of the 33-3 rats used in this experiment had clinical arthritis, but all had chronic diarrhea. Administration of dexamethasone decreased diarrhea in 33-3 rats (Table 1, study 1). Consistent with this observation, gross examination of MLN and the colon at autopsy demonstrated reduced MLN hyperplasia, and weight/length ratio of the colon in dexamethasone-treated 33-3 rats (Table 2, study 1). MLN weight was also reduced in dexamethasone-treated nontransgenic F344 rats. Histological examination of the colonic mucosa revealed chronic inflammation in 33-3 rats. However, the histological score of lesions was unchanged in dexamethasone-treated 33-3 rats, as compared to vehicle-treated rats (Table 2, study 1). An inhibitory effect of dexamethasone was detected in the colon on several markers linked to gut inflammation, *i.e.* MPO enzymatic activity (Table 3, study 1), and IFN-γ, IL-1β and CD3 transcripts evaluated by semi-quantitative RT-PCR (Figure 3A). However, neither iNOS enzymatic activity (Table 3, study 1), nor iNOS mRNA expression (Figure 3A), examined in the colonic mucosa were modified in dexamethasone-treated 33-3 rats.

L-NIL decreases iNOS activity, and aggravates the spontaneous inflammatory disease of HLA-B27 trasngenic rat. We further addressed the role played by iNOS in the disease process of HLA-B27 transgenic rat, by administering L-NIL (10 $mg.kg^{-1}.day^{-1}$) or

Table 1
Effect of dexamethasone (study 1) and L-NIL (study 2) on the scores of diarrhea and arthritis in HLA-B27 transgenic rats

	Week of treatment				
Group of rats	0	1	2	3	4
Study 1					
Score of diarrhea					
33-3: vehicle (n = 6)	1.7 ± 0.5	2 ± 0.6	2.3 ± 0.6	2.5 ± 0.8	2.9 ± 0.8
33-3: dexamethasone (n = 6)	2.3 ± 0.3	2.5 ± 0.4	2.1 ± 0.4[a]	1.6 ± 0.2[b]	1.3 ± 0.5[a]
Study 2					
Score of diarrhea					
33-3: vehicle (n = 8)	1.6 ± 0.3	2 ± 0.2	1.9 ± 0.1	2 ± 0.1	
33-3: L-NIL (n = 8)	2.3 ± 0.2	2.4 ± 0.2	2.3 ± 0.2	2.1 ± 0.2	
Arthritic score					
33-3: vehicle (n = 8)	0.5 ± 0.5	1.1 ± 0.7	1 ± 0.6	0.9 ± 0.5	
33-3: L-NIL (n = 8)	0.8 ± 0.4	1.6 ± 0.5	2 ± 0.5	2 ± 0.6	

Study 1: HLA-B27 transgenic rats of the 33-3 line with established disease received dexamethasone (0.25 $mg.kg^{-1}.day^{-1}$) or vehicle for 4 weeks. Dexamethasone- and vehicle-treated nontransgenic F344 littermates had no diarrhea. [a] $P = 0.015$ dexamethasone *vs* vehicle: week 2 and week 4 minus week 0. [b] $P < 0.01$ dexamethasone *vs* vehicle: week 3 minus week 0.
Study 2: HLA-B27 transgenic rats of the 33-3 line with established disease received L-NIL (10 $mg.kg^{-1}.day^{-1}$) or vehicle for 3 week. L-NIL- and vehicle-treated nontransgenic F344 littermates had no detectable diarrhea or arthritis.

Table 2
Effect of dexamethasone (study 1) and L-NIL (study 2) on MLN weight, colon weight/length and quantitative histological score of HLA-B27 transgenic rat colon

Group of rats	MLN wt (mg)	Colon wt/length (mg/cm)	Histologic score
Study 1			
33-3: vehicle (n = 6)	1,180 ± 184	239 ± 31	16.5 ± 1
33-3: dexamethasone (n = 5)	413 ± 88[a]	158 ± 12[b]	17 ± 1.1
Ntg: vehicle (n = 4)	126 ± 26	98 ± 19	0
Ntg: dexamethasone (n = 4)	27 ± 5[c]	90 ± 1	0
Study 2			
33-3: vehicle (n = 8)	863 ± 49	217 ± 22	8.3 ± 1.5
33-3: L-NIL (n = 8)	1055 ± 69[d]	224 ± 18	11.5[e] ± 0.4
Ntg: vehicle (n = 4)	188 ± 34	99 ± 9	1.9 ± 1.5
Ntg: L-NIL (n = 4)	163 ± 36	117 ± 3	0

HLA-B27 transgenic rats of the 33-3 line and nontransgenic (Ntg) F344 rats were treated with dexamethasone (0.25 $mg.kg^{-1}.day^{-1}$) or vehicle for 4 weeks (study 1), or with L-NIL (10 $mg.kg^{-1}.day^{-1}$) or vehicle for 3 weeks (study 2). MLN weight and weight/length of colon were measured at sacrifice.
Study 1: 33-3: dexamethasone *vs* vehicle; [a] $P < 0.005$; [b] $P < 0.04$
Ntg: dexamethasone *vs* vehicle; [c] $P < 0.03$
Study 2: 33-3: L-NIL *vs* vehicle: [d] $P < 0.04$; [e] $P < 0.03$.

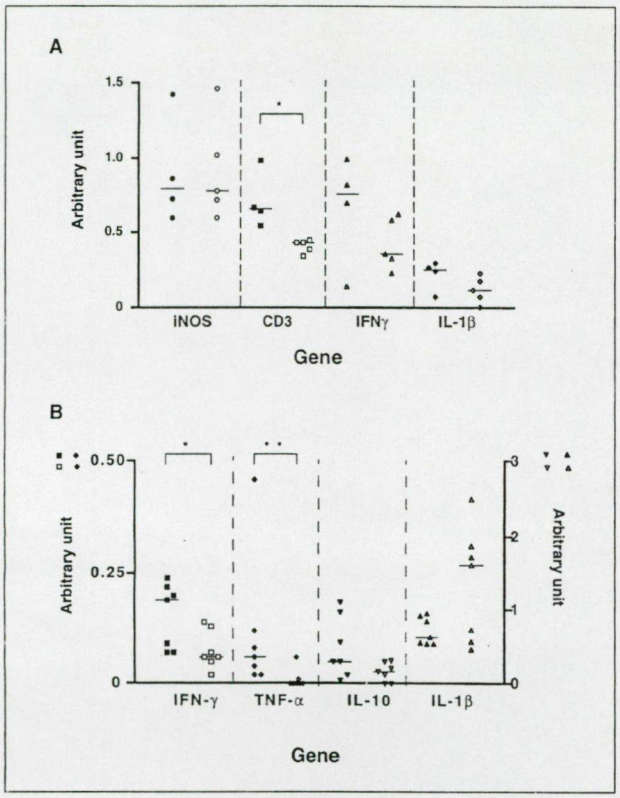

Figure 3
Semi-quantitative RT-PCR evaluation of iNOS and cytokines transcripts in the colonic mucosa from HLA-B27 transgenic rats. Individual rat values and medians are shown.
A: *study 1:* iNOS, CD3, IFN-γ, and IL-1β transcripts in the colonic mucosa from vehicle-treated B27 transgenic rats (close symbols), and from dexamethasone-treated B27 transgenic rats (open symbols). * $P < 0.05$, dexamethasone- *vs* vehicle-treated groups. In the colon of dexamethasone- (n = 4) or vehicle-treated (n = 2) nontransgenic F344 littermates, trancripts for iNOS, IFN-γ or IL-1β were not detected and transcripts for CD3 were negative or ≤ 10-fold below the level of detection achieved in B27 transgenic rats. **B:** *study 2:* IFN-γ, TNF-α (left scale), and IL-10, IL-1β (right scale) transcripts in the colonic mucosa from vehicle-treated (close symbols), and from L-NIL-treated B27 transgenic rats (open symbols). * $P < 0.05$, L-NIL- *vs* vehicle-treated group. ** $P < 0.01$, L-NIL- *vs* vehicle-treated group. In the colon of L-NIL- (n = 2) or vehicle-treated (n = 3) nontransgenic F344 littermates, trancripts for IFN-γ, TNF-α, or IL-1β were not detected, and transcripts for IL-10 were either negative or < 7-fold below the average level of detection in vehicle-treated B27 transgenic rats.

vehicle for 3 weeks to 33-3 rats with established disease (study 2). In this experiment, both diarrhea and arthritis were present. Administration of L-NIL had no detectable effect on diarrhea (Table 1, study 2); arthritis was exacerbated in the L-NIL-treated group, as compared to the vehicle-treated group although the difference was not statistically significant (Table 1, study 2). Evidence for a detrimental effect of L-NIL on the course of inflammatory disease in 33-3 rats was further provided by gross organ examination: MLN hyperplasia was increased in L-NIL-treated 33-3 rats, as compared to vehicle-treated group, whereas the colon weight/length ratio was no different between the two groups (Table 1, study 2). Finally, histological evaluation of the colon demonstrated increased lesions in L-NIL-treated 33-3 rats, as compared to the vehicle-treated group (Table 2, study 2). Variations in MLN weight, histological score, MPO and iNOS activity of vehicle-treated 33-3 groups between study 1 and 2 (Tables 1 and 2) merely reflects the difference in the overall degree of gut inflammation between the 2 studies.

Table 3
Effect of dexamethasone (study 1) and L-NIL (study 2) on MPO and iNOS enzymatic activities in the colon of HLA-B27 transgenic rats

Group of rats	MPO (mU/g of colon)	iNOS (pmol. $min^{-1}.mg^{-1}$ protein)
Study 1		
33-3: vehicle (n = 6)	1,451 ± 455	2.2 ± 0.6
33-3: dexamethasone (n = 5)	475 ± 112	2.2 ± 0.5
Ntg: vehicle (n = 4)	690 ± 126	0
Ntg: dexamethasone (n = 4)	76 ± 25[a]	0
Study 2		
33-3: vehicle (n = 8)	477 ± 113	1.4 ± 0.4
33-3: L-NIL (n = 6)	649 ± 212	0.4 ± 0.1[b]
Ntg: vehicle (n = 4)	319 ± 70	0.2 ± 0.1
Ntg: L-NIL (n = 4)	351 ± 105	0.1 ± 0.2

HLA-B27 transgenic rats of the 33-3 line and nontransgenic (Ntg) F344 rats were treated with dexamethasone (0.25 $mg.kg^{-1}.day^{-1}$) or vehicle for 4 weeks (study 1), or with L-NIL (10 $mg.kg^{-1}.day^{-1}$) or vehicle for 3 weeks (study 2). MPO and iNOS enzymatic activity were measured in samples of colon harvested at sacrifice.
[a] $P < 0.05$, Ntg: dexamethasone *vs* vehicle *(study 1)*.
[b] $P = 0.05$, 33-3: L-NIL *vs* vehicle *(study 2)*.

Influence of L-NIL treatment on cytokine production in the colon of B27-transgenic rats. Inhibition of iNOS enzymatic activity by L-NIL was confirmed in the colon, whereas MPO activity was not significatively modified by L-NIL treatment (Table 3, study 2). RT-PCR analysis detected comparable levels of CD3, iNOS, and IL-10 transcripts in the colonic mucosa of 33-3 rats treated with L-NIL or with vehicle (Figure 3B, and data not shown). As regards cytokine transcripts, a significant decrease was observed for IFN-γ, and TNF-α in L-NIL-treated 33-3 rats, in comparison to vehicle-treated group (Figure 3B). In contrast, IL-1β mRNA transcripts were increased in the L-NIL-treated group, as compared to vehicle-treated 33-3 rats, but this difference did not reach statistical significance. No consistent difference in pathology or MPO activity was detected between a subgroup of 4 rats with high IL-1β transcript values and the 3 remaining rats with lower values, among L-NIL-treated 33-3 rats (data not shown).

DISCUSSION

Expression of iNOS was detected in two prominent target organs of HLA-B27 transgenic rat inflammatory disorder, namely the colonic mucosa and joint. Induction of iNOS was reproducibly detected in the hip cartilage of 33-3 rats, even though arthritis was not observed at this site. In contrast, iNOS was not detected in the synovium, even from arthritic joints, which

may suggest that chondrocytes are specifically triggered to produce iNOS in this model. Conceivably, a common mechanism may result in iNOS expression in both gut and joint, since inflammation of these two organs is closely linked in the HLA-B27 transgenic rat [2]. In the colonic mucosa, iNOS expression was predominantly localized in the epithelial cells, with only limited expression in the inflammatory cells infiltrating the lesions. Among possible triggers of iNOS expression, cytokines produced locally in the setting of chronic inflammation and luminal bacterial products, such as LPS, are likely candidates [18, 19].

We examined *in vivo* the role played by iNOS in the HLA-B27 transgenic rat model by administering dexamethasone, which may prevent the expression of iNOS [20] and the putative iNOS inhibitor, L-NIL. Dexamethasone treatment improved chronic diarrhea, and reduced MLN hyperplasia, and colon weight/length ratio in 33-3 rats with established disease. It also decreased MLN weight and MPO activity in the colon of nontransgenic control F344 rats. All these changes are suggestive of an anti-inflammatory effect. Nevertheless the efficacy of dexamethasone on 33-3 gut lesions was not confirmed when examined histologically. We also observed a lack of influence of dexamethasone on iNOS expression. This latter result is consistent with a study carried out in human inflammatory bowel disease [21], although it is at variance with reports showing inhibition by corticosteroids of cytokine- and LPS-mediated iNOS induction [11, 14]. Such inhibitory effects of dexamethasone are thought to involve inhibition of nuclear factor-kappaB (NF-κB) activation, which is important for induction of the iNOS gene [22]. However, inhibition of NF-κB by dexamethasone is not uniformly observed [23], and dexamethasone-insensitive pathways of iNOS induction, other than NF-κB have been described in epithelial cells [24]. In the present study, the decrease of CD3 and cytokine transcripts in colon, observed after dexamethasone treatment, was not associated with a striking inhibition of gut inflammation. This result is consistent with our previous work, in which administration of IL-10 to 33-3 rats failed to improve colonic inflammation, although it did reduce CD3, IFN-γ, TNF-α, and IL-1β, and conversely increased iNOS expression in the colon (5; M. Breban and D. Lamarque, unpublished results). Altogether, these results argue for a dispensable role of this set of pro-inflammatory cytokines in perpetuating the disease process and in the induction of iNOS. It is likely that other mediators are critically involved in the sustained colonic inflammation of HLA-B27 transgenic rats. However, since dexamethasone had no effect on iNOS, this study could not properly address the role of iNOS in this model of colitis.

In a further study, L-NIL was used as a selective inhibitor of iNOS [12]. Enzymatic inhibition of iNOS was achieved with this compound. L-NIL-treatment was associated with aggravation of several markers of disease severity, *i.e.* arthritis, MLN hyperplasia, and colonic histological inflammation. Another noticeable effect of L-NIL administration was a decrease of several cytokine transcripts in the colon, *i.e.* IFN-γ and TNF-α. However, a trend towards increased IL-1β was conversely observed in the same tissue. Hence, this result suggests that NO produced by epithelial cells in the inflammed colonic mucosa results in either stimulatory (IFN-γ, TNF-α) or inhibitory (IL-1β) effects on cytokine production. Depending on the site of production, the amount produced, and the targets within the local environment, NO has indeed been shown to display opposite effects, including those on cytokine production [25-27]. In the colitis of HLA-B27 transgenic rats, it is not yet known which cells produce specific cytokines, and NO may exert an effect on different target cells, such as T cells, macrophages or epithelial cells, resulting in distinct variations of cytokine transcripts. In any case, stimulation of IL-1β transcription may provide an explanation for the damaging consequence of iNOS inhibition in the present model. In a study by Aiko and colleagues, two other NO synthase inhibitors, *i.e.* aminoguanidine and L-NAME, were used in this HLA-B27 transgenic rat model [28]. The major effects of NO synthase inhibitors reported by these authors were decreased MPO activity in the colonic mucosa and decreased bowel mucosal permeability. Increased colonic lesions were not described. However, no quantitative histological analysis of colonic ulceration and infiltration by inflammatory cells was performed. Hence, differences with the present study could be related to different inhibitory activity of the inhibitors used in their study, as compared to L-NIL, or to a differences in study protocols.

Induction of iNOS has been described in a variety of inflammatory diseases, in humans and in animal models, including colitis, arthritis and psoriasis which are features of the HLA-B27 transgenic rat model [25, 29, 30]. Interestingly, expression of iNOS in human ulcerative colitis was shown to predominate in epithelial cells rather than in infiltrating leukocytes of the colonic mucosa, a finding similar to our observation in HLA-B27 transgenic rat [31]. Investigating the potential implication of iNOS induction in the pathogenic mechanism of this model is of considerable interest, since iNOS has been shown to exert both protective and detrimental effects on several inflammatory processes, including chronic gut inflammatory situations and arthritis [9, 32]. NO from iNOS seems to contribute to tissue destruction in acute inflammatory disease through the generation of its cytotoxic derivative peroxynitrite, when produced by macrophages and neutrophils in combination with oxygen free radicals [33]. Such a consequence is less likely to happen if NO production takes place in epithelial cells, such as in chronic colitis of HLA-B27 transgenic rat. Local production of NO in the epithelium may rather display antibacterial effects that could be beneficial in this model, in which gut bacterial flora is critically involved in the induction of both gut and joint inflammation [2]. NO may also display immunoregulatory activities which are mediated by the modulation of cytokine production and apoptosis of effector T cells, resulting in a decrease of Th1-dependant autoimmune diseases [9]. Regarding a putative immu-

noregulatory mechanism, it is worth noting that IL-1β, which has been repeatedly implicated in the pathogenesis of IBD was rather increased in the colon after L-NIL treatment [34-36].

Our results provide a strong suggestion that iNOS is protective, at least in colitis and presumably in arthritis in the HLA-B27 transgenic rat model. To our knowledge, this is the first evidence that NO is protective in a spontaneous animal model of chronic colitis or arthritis. It will be important to consider this result when discussing the therapeutic prospect of selective iNOS inhibitors in chronic inflammatory disorders [25, 37].

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Lymphocytic infiltration and expression of inducible nitric oxide synthase in human duodenal and colonic mucosa is a characteristic feature of ankylosing spondylitis. J Rheumatol. 2003; 30: 2428-2436.

Lymphocytic Infiltration and Expression of Inducible Nitric Oxide Synthase in Human Duodenal and Colonic Mucosa Is a Characteristic Feature of Ankylosing Spondylitis

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ABSTRACT. ***Objective.*** In patients with ankylosing spondylitis (AS), inflammatory processes have been detected in the ileal and colonic mucosa. The inducible isoform of nitric oxide synthase (iNOS) may be expressed early in the inflammatory process. We investigated iNOS activity and lymphocytic infiltration in the duodenum and colon in patients with AS and ulcerative colitis compared with controls.

Methods. Gastroscopy with duodenal biopsies and/or colonoscopy with biopsies were conducted in 42 patients with AS treated or not treated with nonsteroidal antiinflammatory drugs (NSAID), in 15 with ulcerative colitis, and in 46 controls. Lymphocytic infiltration in the lamina propria and intraepithelial infiltration were quantified by histological score. iNOS expression was assessed by immunohistochemistry with monoclonal antibodies, and iNOS activity was determined by radiochemical assay.

Results. Endoscopic examination of the gastroduodenal or colonic mucosa did not reveal macroscopic lesions in the AS patients. In the duodenum, mucosal lymphocytic infiltration was found in 83.3% of the AS group compared to 48.6% of controls ($p = 0.02$), and was independent of the NSAID intake. Intraepithelial lymphocyte infiltration was increased in both duodenum and colon in AS patients compared to controls. iNOS activity in duodenum and colon and expression of iNOS protein in lamina propria inflammatory cells was increased in AS patients compared to controls.

Conclusion. Lymphocytic infiltration and iNOS expression and activity were detected in duodenal and colonic mucosa from patients with AS. Such findings may indicate an inflammatory process in the small intestine and colon of patients with AS. (J Rheumatol 2003;30:2428–36)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS NITRIC OXIDE
COLITIS DUODENUM INFLAMMATION

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Histologic features of inflammation have been described in ileal and colonic mucosa from patients with ankylosing spondylitis (AS). Up to 56% of those patients have exhibited lymphocytic infiltration in the colonic lamina propria, suggesting inflammatory bowel disease[1,2]. Inflammation of the gut has been proposed to be linked to the pathogenesis of AS by provoking a break in the epithelial barrier, permitting luminal antigens, especially bacteria, to penetrate into the mucosa and subsequently to activate an immune process[1]. One consequence of such a process involving epithelial dysfunction would be the release of local bacterial products that cause expression of mediators and markers of inflammation.

Expression of inducible nitric oxide synthase (iNOS) could be such a marker, as it appears in the initial phase of inflammation in many animal models of gut inflammation[3]. Thus, after systemic low dose administration of endotoxin, iNOS is expressed within 3 hours in duodenal or colonic tissue and epithelial cells and is associated with epithelial cytotoxicity and microvascular leakage of albumin[4-7].

Expression of iNOS in the colon is also observed as an early event in experimental colitis induced by trinitrobenzene sulfonic acid in the rat and in an HLA-B27 transgenic model of AS[8,9]. Induction of iNOS is stimulated by the proinflammatory cytokines interferon-γ (IFN-γ) and interleukin 2 (IL-2)[10,11], whereas IL-10 is known to modulate the inflammatory response and reduce iNOS expression[12].

To explore the possibility that gut inflammation in patients with AS extends to other regions of the gastrointestinal (GI) tract, we investigated the infiltration of lymphocytes into the epithelium and lamina propria, along with iNOS activity and expression in mucosa of both the duodenum and the colon in patients with AS. These factors in the duodenal and colonic mucosa were compared with control patients or patients with ulcerative colitis, respectively. Moreover, the expression of mRNA for other markers of inflammation, the cytokines IFN-γ, IL-2, and IL-10, was also evaluated in duodenum from patients with AS and control patients.

MATERIALS AND METHODS

Patients. Patients who had dyspepsia, abdominal pain, or diarrhea were referred for esophago-gastroduodenoscopy or colonoscopy in the Henri Mondor Hospital endoscopy unit and were considered for inclusion in the study. Patients with concomitant known intestinal or significant hepatic, respiratory, cardiovascular, or renal disease or malignancy, or those taking medications other than nonsteroidal antiinflammatory drugs (NSAID) were excluded. Patients treated for *Helicobacter pylori* infection were excluded.

Forty-two patients with AS were recruited from rheumatology departments at Henri Mondor Hospital and Cochin Hospital over a 36 month period. All patients were diagnosed with AS, according to the New York criteria, and all except 3 had the HLA-B27 antigen[13]. Axial involvement was present in all patients. According to the exclusion criteria, none of the AS patients were treated with sulfasalazine. Patients were divided into 2 groups: 14 patients who had used NSAID at least once per week in the previous month and 28 patients whose NSAID treatment was interrupted in the previous week for the purpose of the study.

A group of 15 patients having clinically active ulcerative colitis not associated with AS or peripheral arthritis or other inflammatory diseases were also recruited.

Finally, 46 patients were selected for the control group from patients referred to our endoscopy unit for polyp screening or dyspepsia. Patients with GI disease or taking antisecretory drugs were excluded from these groups. Nine of the control patients had taken NSAID for osteoarthritis.

All patients signed an informed consent approved by the institutional ethics committee.

Tissue samples. All tissues evaluated in this study were obtained from endoscopic biopsy specimens. In patients having esophago-gastroduodenoscopy, 2 biopsies were taken from the antrum and 6 from the duodenum for histology. In patients having colonoscopy, 6 samples were taken from the ascendant colon. For measurement of iNOS and mRNA analysis, 8 additional biopsies obtained from the duodenum or colon were snap-frozen in liquid nitrogen.

Histological inflammation grading. After fixation in buffered formalin, tissues were embedded in paraffin, sectioned, and stained with hematoxylin, eosin, and safran. All histological sections were reviewed by a single GI pathologist (JTVN), who was blinded to the other characteristics studied. Sections were scored for lymphocytic infiltration in the lamina propria (absent: 0, mild: 1, moderate: 2, severe: 3) or the intraepithelial lymphocytic infiltration (for duodenum: < 20%: 0, from 20% to ≤ 50%: 1, more than 50%: 2; for colon: < 10%: 0, 10% to 30%: 1, > 30%: 2). *H. pylori* infection was diagnosed by examination of antral mucosal samples stained with cresyl fast violet.

Immunohistochemistry. An antigen retrieval method was performed on 5 µm deparaffinized sections of duodenum or colon, using microwave oven heating (3 × 5 min) in 6 mM citrate buffer, pH 6. Sections were then incubated 10 min in 3% hydrogen peroxide to block endogenous peroxidase activity, followed by 1 h with anti-iNOS monoclonal antibody, dilution 1/40 (Transduction Laboratories, Lexington, KY, USA). Cross-reactivity of these antibodies to human iNOS has been described by immunohistochemistry[14]. This antibody has previously been used to determine iNOS expression in gastric mucosa from patients with *H. pylori* infection[15]. That study has shown that the immunostaining was in agreement with the mRNA expression of iNOS. A 3-step indirect process, based on avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) and 3,3-diaminobenzidine (Sigma, St-Quentin Fallavier, France) as chromogen, was applied to reveal staining as a brown precipitate. Negative controls were obtained by omitting the primary antibody and by staining after incubating the antibody for 2 h with the iNOS peptide provided by Transduction Laboratories. The extent of the immunoreactivity was quantified by a score ranging from 0 to 3 in epithelium and in lamina propria. The score was: 0 when < 10% of the cells were stained, 1 when 10% to 30% were stained, 2 when 30% to 50% were stained, 3 when > 50% of the cells were stained.

Determination of iNOS enzymatic activity. Activity of iNOS was estimated by measuring the conversion of L-[^{14}C]-arginine monohydrochloride to L-[^{14}C]-citrulline, as described[7]. Briefly, duodenal or colonic sample was homogenized in HEPES buffer containing protease inhibitors. After centrifugation (10,000 g, 30 min, 4°C), an aliquot of supernatant was added to prewarmed buffer containing (final concentration) potassium phosphate (50 mM, pH 7.4), valine (50 mM), $MgCl_2$ (1 mM), $CaCl_2$ (200 µM), DL-DTT (1 mM), L-citrulline (1 mM), NADPH (0.3 mM), FAD (3 µM), FMN (3 µM), BH4 (3 µM), L-[^{14}C]-arginine monohydrochloride (15.5 nM; Amersham, Les Ulis, France) and incubated 30 min at 37°C. The incubation was terminated by the binding of arginine, by addition of a 1:1 suspension of Dowex (AG 50W-8; Sigma) in water. After centrifugation, supernatant was taken for scintillation counting. Activity of iNOS was that which was inhibited by *in vitro* incubation with the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA; 300 µM; Sigma) but not with EGTA (1 mM), and was expressed as pmol·min^{-1}·mg^{-1} protein[16].

Reverse transcription-polymerase chain reaction (RT-PCR). Expression of IL-2, IL-10, and IFN-γ in the duodenal mucosa was estimated by mRNA detection with RT-PCR, and was semiquantified by comparison with actin mRNA expression. Total RNA was isolated with the TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and reverse transcribed into complementary DNA (cDNA) using AMV reverse transcriptase (Promega, Madison, WI, USA). Amplification was carried out in a 100 µl volume containing 250 ng cDNA, 125 µM of each 2'-deoxynucleoside 5'-triphosphate, 2 U of Taq DNA polymerase (Appligene, Illkirch, France), 50 ng of each primer, and 10 µl of 10 × PCR buffer. The following primer sequences were used: IL-2 sense 5'-GTC ACA AAC AGT GCA CCT AC-3', antisense 5'-CCC TGG GTC TTA AGT GAA AG-3'; IL-10 sense 5'-AAA TTT GGT TCT AGG CCG GG-3', antisense 5'-GAG TAC TCT GGT TGG TCT TC-3; IFN-γ sense 5'-GCA GAG CCA AAT TGT CTC CT-3', antisense 5'-ATG CTC TTC GAC CTC GAA AC-3'; ß-actin sense 5'-GGG TCA GAA GGA TTC CTA TG-3', antisense 5'-GGT CTC AAA CAT GAT CTG GG-3'. Amplification conditions consisted of 3 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 57°C, extension at 72°C for 30 s, and by final elongation at 72°C for 10 min. Fifty microliters of PCR product were electrophoresed on agarose gel. Negative pictures of the gels were taken for quantitation with a Personal Densitometer using the Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA, USA). Data are expressed in arbitrary units as the ratio between optical density for the specific cDNA and the one for ß-actin on the same sample.

Statistics. Quantitative variables were expressed as mean ± SEM. Contingency analysis was performed using the Fisher exact test. Single comparisons were made by the Student t test or by Wilcoxon nonparametric test where appropriate. Links between the different histological scores were by Kruskal-Wallis nonparametric or parametric analysis of variance (ANOVA) test. Multiple comparisons between groups were performed using one-way ANOVA. P values < 0.05 were considered significant.

RESULTS

Clinical results and H. pylori status. There were no significant differences in the mean ages (± SEM) of the groups: 41.9 ± 2, 42.9 ± 5, 51.0 ± 3, and 52.0 ± 2 years for AS untreated, AS NSAID users, ulcerative colitis groups, and controls taking NSAID or not, respectively. Diarrhea, defined as regular episodes of diarrhea with stool frequency > 4 per day, was observed in 7 (17%) patients with AS and in all patients with ulcerative colitis.

Endoscopic examination of the gastroduodenal or colonic mucosa did not reveal any macroscopic lesion in AS or control patients. Colonic examination of patients with ulcerative colitis revealed pancolitis in 5 patients, left-side colitis in 10 patients. No patient showed dysplasia or toxic megacolon.

H. pylori status, analyzed in patients who had esophagogastroduodenoscopy, was positive in 10 of 24 (42%) patients with AS who were non-NSAID users, in 4 of 12 (33%) patients with AS who were NSAID users, and in 23 of 35 (66%) controls.

Histological characteristics. Duodenum. The infiltration scores of lymphocytes in the lamina propria of the duodenum and of intraepithelial lymphocytes are shown in Tables 1 and 2, respectively. Lymphocytic infiltration in the duodenal lamina propria was found more frequently and was more intense in patients with AS, both treated or not treated with NSAID, as compared to the corresponding control group (Table 1). Intraepithelial lymphocytosis in the duodenum was also found more frequently and was more marked in AS patients treated or not with NSAID than in controls (Table 2).

In patients with AS, the percentage of *H. pylori* positive patients that showed lymphocytic infiltration in lamina propria (79%) or increased intraepithelial lymphocytes (86%) was not different compared to that of *H. pylori* negative AS patients (86% and 64%, respectively).

Histological characteristics. Colon. In the colon, the frequency or the mean grade of lamina propria infiltration by lymphocytes was not different between AS patients treated with NSAID or untreated and the control patients (Table 1).

The intraepithelial lymphocytosis in colon was more frequently observed and the mean score was significantly higher in AS patients both treated and not treated with NSAID than in control patients (Table 2).

In patients with ulcerative colitis, the frequency and the mean grade of lymphocytic infiltration in lamina propria or intraepithelial lymphocytes was increased compared to the control group (Tables 1 and 2).

Immunohistochemistry. Duodenum. As shown in Figure 1 and Table 3, iNOS staining in the duodenum was more frequently detected and the mean grade was higher in lamina propria inflammatory cells from patients with AS, whatever the NSAID user status, than in controls. The frequency and the mean grade of iNOS staining in epithelial cells, however, was not different in the untreated or treated AS groups from that in the control group (Table 4). There was a correlation between the iNOS immunostaining score and the score of lymphocytic infiltration in the duodenal lamina propria ($p < 0.02$).

Immunohistochemistry. Colon. In the colon, iNOS staining was observed more frequently and with a higher score in lamina propria from AS patients (NSAID treated or not) than in control patients (Table 3, Figure 2). In contrast, iNOS staining in colonic epithelial cells was found more frequently and with higher score only in AS patients who were not treated with NSAID, as compared to control patients (Table 4). The iNOS staining in colonic lamina

Table 1. Infiltration score of lymphocytes in lamina propria of the duodenum and colon in biopsies from the control group, patients with AS either treated with NSAID or untreated, and patients with ulcerative colitis.

	n	Grade ≥ 1 (%)	Mean Grade (0–3 scale)
Duodenum			
Control	28	17 (48.6)	0.50 ± 0.1
AS untreated	24	20* (83.3)	0.92 ± 0.1**
AS treated with NSAID	12	10* (83.3)	0.92 ± 0.1**
Colon			
Control	12	4 (33.3)	0.38 ± 0.15
AS untreated	12	5 (41.7)	0.75 ± 0.14
AS treated with NSAID	11	3 (27.3)	0.63 ± 0.1
Ulcerative colitis	10	10* (100)	2.56 ± 0.16**

* $p < 0.05$ vs corresponding control group, Fisher exact test. ** $p = 0.01$ vs corresponding control group, Kruskal-Wallis nonparametric ANOVA.

Table 2. Infiltration score of intraepithelial lymphocytes in the duodenum and colon from biopsies from the control group, patients with AS treated with NSAID or untreated, and patients with ulcerative colitis.

	n	Grade ≥ 1 (%)	Mean Grade (0–3 scale)
Duodenum			
Control	28	8 (28.6)	0.42 ± 0.1
AS untreated	24	15* (62.5)	0.88 ± 0.2**
AS treated with NSAID	12	11* (91.6)	1.11 ± 0.2**
Colon			
Control	12	1 (8.3)	0.08 ± 0.1
AS untreated	12	6* (50.0)	0.63 ± 0.2**
AS treated with NSAID	11	6* (54.5)	0.59 ± 0.2**
Ulcerative colitis	15	4* (26.7)	0.40 ± 0.1**

* $p < 0.05$ vs corresponding control group, Fisher exact test. ** $p < 0.05$ vs corresponding control group, Kruskal-Wallis nonparametric ANOVA.

propria or in epithelial cells was found more frequently and was more marked in the ulcerative colitis group than in the control group (Tables 3 and 4, Figure 2).

iNOS enzyme activity in duodenum and colon. The iNOS activity was significantly increased in the duodenum from AS patients, both treated or not, compared to control patients (Table 5).

In the colon, the iNOS activity was significantly higher in patients with AS (treated with NSAID or not) or in patients with ulcerative colitis than in control patients (Table 5).

An association between the immunostaining and the iNOS activity ($p < 0.01$) was detectable in 28 of 31 (90%) AS patients who had positive immunostaining in lamina propria of the duodenum. In contrast, iNOS activity was not detectable in 4 of the 5 patients with negative immunostaining.

Expression of IL-2, IL-10, and IFN-γ in duodenal mucosa. The expression of cytokines IL-2, IFN-γ, and IL-10 in the duodenal mucosa was estimated by mRNA detection with RT-PCR, and was semiquantified by comparison with actin mRNA expression. Thus the ratios of IL-2/ß-actin and IFN-γ/ß-actin in the duodenal mucosa were increased in AS patients treated with NSAID (0.30 ± 0.1 and 1.38 ± 0.4, respectively) or not treated with NSAID (0.19 ± 0.0 and 0.21 ± 0.1, respectively) as compared to the control (0.01 ± 0.0, $p < 0.01$; and 0.00 ± 0.0, $p < 0.01$). IL-10 expression was similar in the 3 groups (0.30 ± 0.1, 0.49 ± 0.1, and 0.13 ± 0.2, respectively).

DISCUSSION

Gut inflammation in association with AS has been described in the ileum and the colon[17-20]. To extend these findings to another gut region using both histological and biochemical approaches, we evaluated both colonic and duodenal inflammation in patients with AS, both those treated with NSAID and those not treated, using both lymphocytic infiltration and expression of iNOS as markers of the inflammatory process.

We observed lymphocytic infiltration in duodenal lamina propria in 83% of patients with AS. Lymphocytic infiltration has been previously described in ileal mucosa from 57% of AS patients, independently of GI symptoms[2]. This higher frequency of histologically detected inflammation in the duodenum in our study could be related to the site of the gut studied, or more likely to the population of patients we evaluated, 95% of whom had axial involvement, known to be associated with inflammation in the gut[20].

However, lymphocytic infiltration in the colonic lamina propria was not found more frequently in AS patients than in the control group. In a previous study with systematic biopsy samples, it was reported that inflammation in the colon occurred less frequently than that in the ileum (40% and 60%, respectively) and that the major number of patients (60%) had inflammation detectable only in the ileum[1,17]. The lack of lymphocytic infiltration in lamina propria of the colon in the present study supports the finding of no colonic macroscopic damage, as assessed by colonoscopy in these patients, who were a relatively homogeneous group with axial involvement without peripheral arthritis. In contrast, in a previous study, 31% of the AS patients exhibited endoscopic features of colonic inflammation[1].

The predominant intestinal involvement of inflammation was not associated with excessive frequency of diarrhea as compared to other studies. We found diarrhea in 17% of AS patients, comparable to that in another study on 354 patients, where diarrhea was reported in 19% of the group[20].

Intraepithelial lymphocytes were increased in the duodenum and colon of AS patients treated with NSAID, but only in the colon in patients not treated with NSAID. Such an increase has not been reported previously, although lymphocytes from the lamina propria located along the epithelial basement membrane of the ileum have been described[21]. Intraepithelial lymphocytes could be involved in AS related inflammation, as lymphocyte expression of the αß heterodimer of the T cell receptor was found in AS patients, similar to that in patients with Crohn's disease[22].

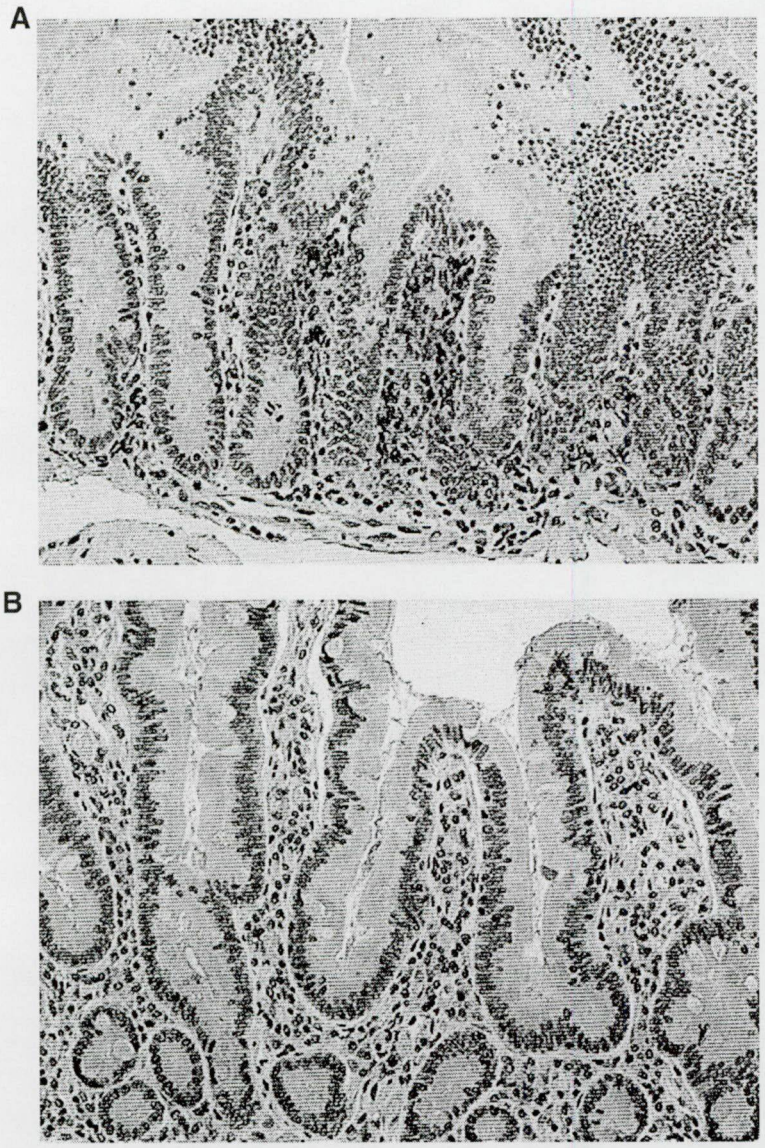

Figure 1. Detection of inducible nitric oxide synthase (iNOS) by immunohistochemistry in the duodenal mucosa. A. Duodenum from patient with AS. The section shows mild infiltration with lymphocytes in the lamina propria and immunohistochemical localization of iNOS as brown staining, mainly in the inflammatory cells (original magnification ×200). B. Normal duodenum from a control patient. The section shows negative iNOS immunostaining (original magnification ×200).

Duodenal lymphocyte infiltration was not modified by the *H. pylori* status. The bacterium usually causes inflammation in the duodenal bulb, especially in patients having ulceration[23]. In our study, biopsies were taken distally in the second part of the duodenum, where no ulcerative damage was seen by endoscopy in any patient[24].

It could be anticipated that histological changes in the gut would be associated with NSAID intake. The group of untreated AS patients did not take NSAID for at least 5 days before the study, although it has been proposed that NSAID intestinal damage may persist for up to several months after discontinuation of drugs[25]. However, the degree and nature of the inflammation that we observed in the duodenum and colon are probably not related to the NSAID. Thus, we found no ulcerative damage or polymorphonuclear infiltration, as usually described with NSAID-related damage, in duodenum from AS or control treated patients. Similarly, other studies involving histological examination of biopsies taken from patients treated with NSAID also did not find duodenal inflammation[23].

Table 3. Immunohistochemical analysis of iNOS in lamina propria of the duodenum and colon in biopsies from the control group, patients with AS treated with NSAID or untreated, and patients with ulcerative colitis.

	n	Grade ≥ 1 (%)	Mean Grade (0–3 scale)
Duodenum			
Control	28	16 (57.1)	0.64 ± 0.1
AS untreated	24	22* (91.7)	1.33 ± 0.3**
AS treated with NSAID	12	9* (75.0)	1.17 ± 1.0**
Colon			
Control	12	3 (25.0)	0.54 ± 0.2
AS untreated	12	12* (100.0)	1.63 ± 0.2**
AS treated with NSAID	11	10* (91.0)	0.95 ± 0.2**
Ulcerative colitis	15	11* (73.3)	1.37 ± 0.3**

* $p < 0.01$ vs corresponding control group, Fisher exact test. ** $p < 0.01$ vs corresponding control group, Kruskal-Wallis nonparametric ANOVA and Dunn's multiple comparison test.

Table 4. Immunohistochemical analysis of iNOS in epithelial cells in duodenum and colon from control group, patients with AS treated with NSAID or untreated, and patients with ulcerative colitis.

	n	Grade ≥ 1 (%)	Mean Grade (0–3 scale)
Duodenum			
Control	28	9 (32.1)	0.36 ± 0.1
AS untreated	24	11 (45.8)	0.58 ± 0.2
AS treated with NSAID	12	6 (50.0)	0.67 ± 0.8
Colon			
Control	12	4 (33.0)	0.92 ± 0.3
AS untreated	12	12* (100.0)	1.81 ± 0.3**
AS treated with NSAID	11	6 (54.4)	1.00 ± 0.3
Ulcerative colitis	15	15* (100.0)	2.63 ± 0.1**

* $p < 0.01$ vs control group, Fisher exact test. ** $p < 0.05$ vs control group by ANOVA.

Consistent with our findings of duodenal or colonic infiltration of lymphocytes in AS patients independent of their NSAID status, mucosal lymphocyte infiltration or increased intestinal permeability have been described in AS patients, either not treated with NSAID or independent of NSAID intake[26-30]. Thus, in a series of 254 AS patients who had undergone ileocolonoscopy, histological inflammation of ileum or colon was not more frequent in patients treated with NSAID (23%) than in those not treated (33%)[20]. In AS patients having ileal and colonic biopsies, 60% of those treated with NSAID had normal biopsies[17].

In our study, the calcium-independent iNOS activity in duodenum and colon was increased in AS patients compared to control patients. These findings of increased iNOS expression or activity were confirmed by immunostaining in both duodenum and colon. Previous studies describe iNOS activity in ulcerative colitis not only in the inflamed area but also in endoscopically normal areas[4,31,32]. Such findings suggest that iNOS is implicated in the moderate stage of mucosal inflammation in inflammatory bowel disease. In our study, the level of iNOS enzyme activity in the colon of AS patients was lower than that seen in patients with ulcerative colitis, which may reflect the lower grade of mucosal inflammation.

The expression of iNOS in lamina propria inflammatory cells may thus be a marker of active intestinal inflammation, which has been previously found in the vicinity of ulcerated lesions in patients with inflammatory bowel disease[33-35]. Such an expression of iNOS in AS patients could reflect a local inflammatory process and not simply the migration of activated inflammatory cells[36,37].

The presence of iNOS, determined by immunochemistry in colonic epithelial cells, was shown in tissue from untreated AS patients. Similarly, iNOS has been found in colonic epithelial cells in patients with ulcerative colitis, and is associated with areas of intense inflammation and clinically active colitis[33,34,38-40]. However, in this study the induction of iNOS in the epithelium or the lamina propria in AS patients was not associated with any overt mucosal damage. Some reports have suggested that the role of iNOS in the pathogenesis of inflammatory bowel disease is not detrimental, but may facilitate the repair process and restore or maintain mucosal integrity[41,42]. Mice deficient in iNOS have more severe damage and delayed healing as compared to controls in the experimental colitis provoked by acetic acid. In addition, the inhibition of NO synthesis by L-N6-(1-iminoethyl)lysine given orally aggravated the severity of the spontaneous colitis observed in rats that are in the transgenic

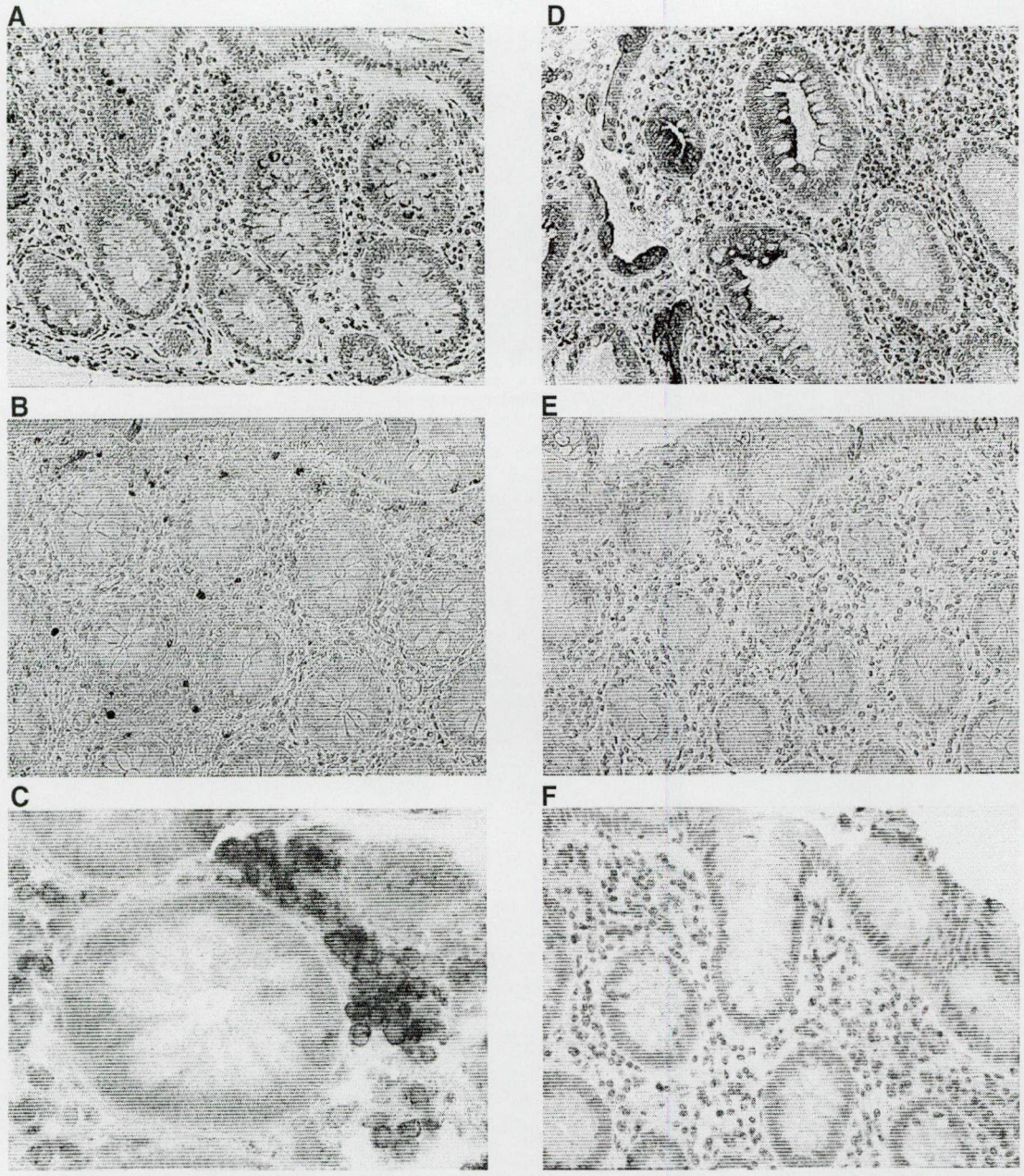

Figure 2. Detection of inducible nitric oxide synthase (iNOS) by immunohistochemistry in the colonic mucosa. A. Colon from patient with untreated AS. The section shows lymphocytic infiltration in the lamina propria and immunohistochemical localization of iNOS as brown staining in the inflammatory cells of the lamina propria and in the crypts and surface epithelium (original magnification ×200). B. Colon from patient with AS treated with NSAID. Immunostaining is present in inflammatory cells of lamina propria. C. Colon from patient with untreated AS. This section shows at higher magnification the cellular location of immunostaining in the lymphocytes of the lamina propria and in the crypt epithelial cells (original magnification ×1000). D. Colon from patient with ulcerative colitis. The section shows ulceration, cryptic abscess, and marked infiltration of inflammatory cells. Immunohistochemical localization of iNOS as brown staining, mainly in the cryptic and surface epithelium (original magnification ×200). Some inflammatory cells are also immunostained. E. Normal colon from control patients. There is no epithelial or inflammatory cell staining (original magnification ×200). F. Colon from patient with untreated AS. Control experiment showing absence of iNOS immunostaining after incubating the antibody 2 h with the iNOS peptide (original magnification ×200).

for HLA-B27 and the human ß$_2$-microglobulin that develop a spontaneous inflammatory disease that resembles human AS in patients who are positive for HLA-B27[9].

iNOS expression was found in both lamina propria and epithelial cells in the colon from AS patients not treated with NSAID. In AS patients receiving NSAID, the iNOS expres-

Table 5. Activity of iNOS in biopsies of the duodenum and colon from the control group, patients with AS treated with NSAID or untreated, and patients with ulcerative colitis.

	n	iNos activity (± SEM) pmol min^{-1} mg $protein^{-1}$
Duodenum		
Control	28	1.8 ± 0.6
AS untreated	24	17.5 ± 3.8*
AS treated with NSAID	12	5.8 ± 6.8**
Colon		
Control	12	2.12 ± 1.0
AS untreated	12	11.5 ± 2.3*
AS treated with NSAID	11	9.0 ± 2.8**
Ulcerative colitis	10	43.47 ± 6.1*

* p = 0.01 vs corresponding control group, Wilcoxon nonparametric test.
** p = 0.05 vs corresponding control group, Wilcoxon noparametric test.

sion was only found in the lamina propria. The immunostaining localization was not superimposed on the lymphocytic infiltration that was found only in the epithelium and not in the lamina propria. The lack of correspondence between these findings is not surprising, as iNOS can be induced in inflammatory cells, especially lymphocytes, involved in the initiation or the regulation of the inflammatory process. The role of these cells is not fully understood and it is possible that they are not consistently localized in the lymphocytic infiltrate[43].

Patients with ulcerative colitis were selected as a control group with lesions since they express iNOS consistently and uniformly in the colon. However, in Crohn's disease, the inflammation is patchy and iNOS expression is not uniform over the colon, thus limiting the reproducibility of the data in such a control group[44].

Expression of iNOS and IFN-γ, determined by mRNA detection with RT-PCR, was found in duodenum from AS patients and not from control patients. Such an association is consistent with previous findings on the induction of iNOS that is dependent on IFN-γ[10]. Lymphocytes infiltrating the mucosa, and particularly intraepithelial lymphocytes, could be the source of IFN-γ in AS. In this study, IL-2 but not IL-10 was also found in duodenum from AS patients, as determined by mRNA detection. The induction of iNOS that is dependent on IL-2 has been observed in natural killer lymphocytes[45]. This finding suggests that lymphocytes in gut mucosa from AS patients may express iNOS.

This study found duodenal and colonic inflammation in association with iNOS induction in more than 80% of patients with AS. These findings suggest a diffuse intestinal inflammatory process in the upper and lower intestine of AS patients. Such events may cause changes in intestinal permeability, permitting exogenous antigen, especially of bacterial origin, to pass through the mucosa and initiate a systemic inflammatory response localized in joints in genetically predisposed patients.

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Successful treatment of steroid resistant ulcerative colitis associated with severe autoimmune hemolytic anemia with oral microemulsion cyclosporin--a brief case report. Am J Gastroenterol. 2003;98:1207-1208.

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Successful Treatment of Steroid Resistant Ulcerative Colitis Associated With Severe Autoimmune Hemolytic Anemia With Oral Microemulsion Cyclosporin—A Brief Case Report

TO THE EDITOR: Since the first publication by Gupta *et al.* in 1984 (1), the beneficial effect of cyclosporin A (CsA) in the treatment of severe ulcerative colitis (UC) has become evident on the basis of placebo-controlled, randomized trials (2, 3). The results of some pilot studies suggest that the *p.o.* microemulsion cyclosporine formulation is at least as effective as *i.v.* administration of CsA (4), CsA has also proved its efficacy in the treatment of pyoderma gangrenosum (5).

Autoimmune hemolytic anemia is a condition rarely associated with UC; the frequency of association is about 1% to 2% (6). We have treated five patients with manifest Coombs positive hemolysis out of more than 400 UC cases observed since 1986 (7). The step-up treatment option of associated autoimmune hemolytic anemia depends on the severity of hemolysis; high dose corticosteroid, either alone or in combination with azathioprine, is the basis of conservative treatment. If these therapeutic modalities remain unsuccessful, splenectomy is indicated first and, in case of failure, total colectomy is the method of choice (8).

There are only two case reports about the beneficial effect of CsA in UC-associated hematological disorders. In the first, a good therapeutic efficacy of combined CsA and fluocortolone was reported in the treatment of ulcerative proctocolitis complicated by severe immune hemolytic anemia (9), whereas in the second, CsA treatment resulted the healing of cyclic neutropenia associated with ankylosing spondylitis and ulcerative colitis (10). In our case, CsA by induction of remission in steroid resistant UC not only saved the young woman from colectomy but also stopped the conventional drug and splenectomy resistant, transfusion requiring, autoimmune hemolysis.

A 35-yr-old woman was admitted to our department in October, 2001, because of a severe flare-up of UC associated with mild jaundice and poor general well-being. Her pancolitis was diagnosed according to standard endoscopic and histological criteria in 1988. An autoimmune Coombs positive hemolytic anemia with cold and warm autoantibodies developed in July, 1998. Twelve units of erythrocyte concentration were transfused. A high dose steroid (methylprednisone, 2 mg/kg *i.v.*) and azathioprine (2 mg/kg) therapy was then introduced. After a 9-month remission achieved with azathioprine, UC and hemolysis reactivated simultaneously. The patient underwent transfusion again, steroid use was restarted (methylprednisone, 1 mg/kg), and danasole was started (400 mg daily) in combination with azathioprine. After a partial response, splenectomy was performed because of the therapy resistant hemolysis in May, 2000. Three months before the patient's present admission to our department, bloody diarrhea started (number of stools, 10–20/day). She had a weight loss of 12 kg until October, 2001. Physical examination showed a moonface caused by steroid therapy, as well as anemia, mild jaundice, and abdominal tenderness at the time of admission. Laboratory values were as follows: erythrocyte sedimention rate 77 mm/h, hemoglobin 84 g/L, hematocrit 27%; serum bilirubin total 22.37 μmol/L, lactate dehydrogenase 819 g/ml, and CRP 68 mg/L. Direct and indirect Coombs tests were both highly positive (++++). As an ultimate preoperative therapeutic possibility, CsA (Sandimmun Neoral; Novartis) was given at a dose of 5 mg per kilogram of body weight (CsA serum concentrations, 81–83 ng/ml, respectively). The activity of UC started to decrease and the hemolysis resolved 1 wk after the initiation of Neoral. Now, 10 months later, the patient is receiving combined azathioprine (2.5 mg/kg) and CsA (4 mg/kg *p.o.*) therapy. Under this therapeutic regimen, there are still mild signs of UC activity but without hemolysis (Coombs test is consequently negative).

CsA suppresses the immune response by inhibiting signal transduction pathways. CsA binds to their intracellular receptors, immunophilins, creating composite surfaces that block the activity of the specific target, calcineurin. Inhibition of the action of calcineurin results in a complete block in the translocation of the cytosolic component of the nuclear factor of activated T cells (11). This effect of CsA seems favorable not only in the induction of remission of UC, but also in the cure of a particular extraintestinal manifestation of UC, namely, pyoderma gangrenosum. Our case suggests that CsA may have beneficial effect in the treatment of another extraintestinal manifestation, namely, autoimmune haemolytic anemia. The reduction of UC activity is surely a key factor of this mechanism, but CsA may also influence the autoimmune hemolysis itself in a favorable manner. The rare association of autoimmune hemolysis and UC probably makes placebo-controlled studies unlikely; therefore, on the basis of these few reported cases, the use of CsA in the treatment of these associated conditions is worth considering.

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Plummer-Vinson Syndrome Associated With Celiac Disease and Complicated by Postcricoid Carcinoma and Carcinoma of the Tongue

TO THE EDITOR: We read with interest two previous reports in the *Journal* on celiac disease–associated Plummer-Vinson syndrome (1) complicated by upper esophageal cancer (2). We report another case in which, in addition to upper esophageal cancer, a carcinoma of the tongue was present.

A 61-yr-old white woman was admitted for evaluation of dysphagia and severe microcytic anemia (Hb 4.9 g/dl) in 2002. Her history was unremarkable except for tuberculosis in 1947, appendectomy in 1968, and cholecystectomy in 1976. Since 1992 she had experienced dysphagia, which forced her to eat slowly and to take small portions of food, but she did not consult a physician. During the 5 wk preceding admission, her dysphagia had worsened so that she was able to swallow liquid foods only, and she lost 10 kg.

At admission she weighed 42 kg and was 160 cm tall. Physical examination revealed pale skin, onychodystrophy, oral fissures, and atrophic glossitis, with an indurated lesion at the left side of the tongue. Laboratory investigations were normal except for severe iron deficiency anemia. A test for fecal occult blood was negative.

Gastroscopy revealed an ulcerating tumor in the upper esophagus, resulting in a 5-cm-long stenosis that could be passed only after endoscopic dilation. Biopsy specimens taken from the tumor showed an invasive, moderately differentiated squamous cell carcinoma grade 2. Biopsy specimens from the antrum and duodenum showed *Helicobacter pylori*–associated atrophic gastritis and villous atrophy of the duodenal mucosa, stage 3C according to the modified MARSH classification (3), corresponding to celiac disease. A test for antiendomysial antibodies was positive. A CT scan showed a stenosing tumor in the upper esophagus that was 1.7 cm in diameter, as well as one enlarged lymph node at the azygo-esophageal angle (stage T3N1M0). Biopsy of the indurated tongue lesion revealed a highly differentiated squamous cell carcinoma. There was no evidence for further lymph node metastases by endosonography and cervical ultrasound.

Initially the patient received two units of packed red blood cells. On a gluten free diet there was a continuous increase in Hb and serum iron. The patient refused surgery but was treated with three cycles of chemotherapy with carboplatin and 5-fluorouracil; both tumors did not respond. Currently the patient is undergoing brachytherapy and teletherapy for her lingual and esophageal cancers, respectively.

This patient had more than one possible reason for severe iron deficiency. The improvement of iron deficiency anemia on a gluten free diet suggests that malabsorption caused by celiac disease (1) was the prime reason. GI symptoms are absent as frequently in the silent or oligosymptomatic form of this disease. In addition to anemia, iron deficiency may be directly linked to the occurrence of esophageal cancer in this patient. The upper esophageal web in Plummer-Vinson syndrome is an established risk factor for the development of esophageal cancer (2, 4). Our patient had chronic dysphagia and long-standing iron deficiency. Despite a lack of direct evidence, it is likely that an upper esophageal web was present in addition to anemia and atrophic glossitis, which is consistent with a retrospective diagnosis of Plummer-Vinson syndrome. Dysphagia was present for 10 yr, and onset of celiac disease presumably occurred many years or decades before. Delay of diagnosis has been recognized as a major risk factor for celiac disease–associated malignancy in large series (4, 5).

To our knowledge, an association between atrophic glossitis or celiac disease and carcinoma of the tongue has not

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Molnar T., **Szepes Z.,** Nagy F., Szenohradszki P., Lonovics J.

Open questions concerning cyclosporine therapy in ulcerative colitis.

Gastroenterology 2004;126:1495-1296

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Open Questions Concerning Cyclosporine Therapy in Ulcerative Colitis

Dear Sir:

We read with interest the article "Randomized, double-blind comparison of 4 mg/kg versus 2 mg/kg intravenous cyclosporine in severe ulcerative colitis"[1] by Gert van Assche et al. in the October 2003 issue of GASTROENTEROLOGY. The authors compared the efficacy and safety of 2 different cyclosporine regimens given by continuous 24-hour infusion followed by oral treatment from the day 8. Some points in this instructive work deserve reflection. First, the title seems somewhat misleading: the basis of the study was not the use of *different doses*, but to attainment of 2 different blood level ranges. The apparent goal of the blood level-based administration (full area under curve [AUC] monitoring) was to achieve the best safety profile during the 7-day infusion therapy. Both ranges (150–250 and 250–350 ng/mL) had been considered therapeutic at the authors' institution before the start of the study. In fact, no significant differences in efficacy or safety were found between the 2 groups, which is not surprising.

A comparison of the frequency of adverse events observed in the "4 mg/kg group" with that in the group examined by Lichtiger et al. in the first famous cyclosporine study[2] reveals appreciable differences in the frequencies of hypertension (9 of 38 vs. 4 of 11) and of paresthesia (3 of 38 vs. 4 of 11, respectively). Lichtiger et al. administered really 4 mg/kg cyclosporine by continuous infusion and attained a higher blood level (mean, 482; range, 339–653 ng/mL). These data suggest that the most important consideration during cyclosporine therapy is to keep the blood level within the narrow therapeutic range.[3] The results of van Assche et al. suggest that a reduced dose could improve the safety of cyclosporine treatment, but the lower and upper ranges within the therapeutic blood levels (237 ± 33 ng/mL vs. 332 ± 43 ng/mL) differed only statistically, and not biologically.

However, why should we compare different blood levels measured during infusion for a certain number of days of therapy? We are not sure that patients displaying fecal urgency on, say 5–10 occasions daily should be treated in this way. The authors write that "most patients with severe colitis feel sick and may not be able to comply with taking Neoral (Novartis) capsules." It is rather difficult to believe that patients who can tolerate 2 endoscopic examinations (and preparations) within 8 days, are not able to take 2 capsules daily. The cyclosporine microemulsion has favorable pharmacokintetic parameters[4] with few associated side effects.[5] It proved effective as first-line therapy at a daily dose of 7–7.5 mg/kg in patients with severe steroid-refractory ulcerative colitis.[6] Our own results presented at the UEGW in Madrid, a lower oral dose of 4 mg/kg cyclosporine may also be effective, with a good safety profile.[7] Complete remission was achieved in 12 of 14 patients after 1 month, and only 1 patient was operated on during the 6-month follow-up period. van Assche et al. used a higher oral dose (8 mg/kg) for 3 months monitoring only the fasting blood level. They did not publish any data on the oral treatment phase, and therefore we do not know whether the therapy was safe enough with this monitoring and what the late colectomy rate was.

The best safety and efficacy profile during oral cyclosporine (Neoral) therapy can be achieved by using 2 sampling times. The 0 and 2-hour (post-dosing) values provide a close correlation with the AUC with an r^2 value of 0.97, while monitoring of the 0-hour blood level alone is of very low predictive value.[8]

By using a markedly lower dose of Sandimmune Neoral (Novartis) as described earlier, we could achieve a therapeutic blood concentration of cyclosporine (between 800 and 1200 ng/mL) 2 hours post-dosing. The 0-hour level was the same (150–300 ng/mL) as that measured in the "2 mg/kg intravenous group," or in the patients treated orally with 8 mg/kg in the study by van Assche et al.

A high peak level may guarantee therapeutic efficacy, but a lower basic level may ensure safety. A beneficial acute therapeutic effect of oral cyclosporine was also observed by van Assche et al. during the treatment of their patients after an anaphylactic reaction due to the infusion. The data mentioned above lead to the conclusion that there is no theoretical advantage of continuous intravenous infusion versus oral therapy.

Finally, another very interesting and important part of their publication was the examination of parameters which may have influenced the effect of cyclosporine. The results of the multivariate analysis are somewhat unexpected. The study by van Assche et al. suggested that smoking has unfavorable effects in the severe ulcerative colitis group. However, other reports,[9] have indicated that smoking influences the course of ulcerative colitis in a favorable manner, while the cessation of smoking can aggravate the symptoms. To return to the mode of therapy, continuous intravenous administration probably prevents the continuation of smoking, and the abrupt withdrawal of nicotine may therefore be responsible for the poorer outcome in patients who smoke.

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Reply. We thank the authors of this letter for their reflections on our recent article in GASTROENTEROLOGY[1] and for sharing their open-label experience with oral cyclosporine in the treatment of severe ulcerative colitis. We would like to reply to some of the concerns raised in this letter.

First, controlled evidence on the use of IV cyclosporine for severe colitis is scarce[2,3] and no controlled trials have been published describing the use of oral cyclosporine in this indication. Therefore, it was and still is logical to investigate the optimal dosing regimen of cyclosporine IV in severe ulcerative colitis (UC). Second, previous studies have demonstrated that taken as a whole patients with severe or fulminant colitis require in-hospital care, not only because of the risk for emergency surgery, but also because they often need intravenous treatment.[4] All the patients in our study were already admitted and on IV therapy, most often steroids, before they were included in the trial. The use of cyclosporine IV, therefore, was not an extra burden in their treatment. The use of Neoral in non-controlled trials may introduce a selection bias toward patients with less severe symptoms. Third, we agree that in our study cyclosporine blood levels in both study arms remained within the therapeutic range. These therapeutic margins however, have been developed for preventing organ rejection and optimal therapeutic blood levels for ulcerative colitis are unknown again due to the paucity of controlled trials. Our study indicates that UC patients whose blood levels have been in the upper end of the margin for transplant patients, have no improved outcome. Fourth, the bioavailablitiy of oral cyclosporine, even in an improved formula such as Neoral, is notoriously unpredictable. The more reliable assays for monitoring oral cyclosporine therapy mentioned by Molnar et al.[5] have only been introduced in clinical practice after our study was initiated and there is no controlled evidence describing their use in UC.

In conclusion, we agree that Neoral can be investigated as an initial treatment for severe colitis. However, given the load of potential bias and ethical considerations, a study investigating the role of oral cyclosporine in this indication can only be designed as a randomized controlled trial with cyclosporine IV as the active comparator. Such a trial needs to be a multicenter effort, since fortunately for the patients acute fulminant colitis has a lifetime incidence of only 20%.

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A Randomized Controlled Trial of Biofeedback That Does Not Have the Power to Conclude

Dear Sir:

In the article recently published by Norton et al.,[1] several methodological issues need to be stressed to avoid misleading interpretation of the results.

The sample size calculations were based on the hypothesis that 67% of individuals would be improved in the group with biofeedback versus only 30% in the standard care group. The expected improvement of the group with biofeedback compared with the standard group may be expressed either as the absolute difference, i.e., 37% (67%–30%) or more regularly, as the relative difference that is 123% (67% representing a 123% increase of 30%). Such large relative differences are not observed for most interventions used in healthcare. As a result, the minimal sample size theoretically required, i.e., 40 subjects in each group is underestimated. Additionally, 3 out of 4 groups do not even reach the minimum size calculated by the authors. The group used as a reference for the statistics included only 29 patients which leads to a power of 75%, i.e., a risk of 25% to fail to show the relative improvement of 123% expected by the authors. Fifty-four percent of the patients were improved in the standard group. This percentage is simply an average, and the confidence intervals, not provided, are probably very large, meaning that this percentage may well be very different in reality than that observed in this study. If the standard care really improved 40% of the patients instead of 30%, with 29 patients in this group, the power of the study was less than 50% with a 50% chance to conclude no statistical difference whereas there really is a difference.

The reference group is not only too small but is probably biased since 30% of subjects were lost to follow up. If those subjects